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EVIDENCE FOR THE MATURATION OF CELLULAR IMMUNE RESPONSES IN EQUINE INFECTIOUS ANEMIA VIRUS-INFECTED PONIES

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
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By

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ABSTRACT OF DISSERATION

EVIDENCE FOR THE MATURATION OF CELLULAR IMMUNE RESPONSES IN EQUINE INFECTIOUS ANEMIA VIRUS-INFECTED PONIES

Equine infectious anemia virus (EIAV) has been used as a model to investigate protective mechanisms against lentiviruses. Unlike other lentiviruses, EIAV replication can be eventually controlled in most infected horses leading to an inapparent carrier state free of overt clinical signs which can last for many years. Maintenance of this carrier state is absolutely dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce the recurrence of disease. However, the immune mechanisms that are responsible for this control of infection are not yet identified. As the resolution of the initial infection is correlated with the appearance of the virus-specific cytotoxic T lymphocytes (CTL), it appears that cellular immune responses play an important role. However, most studies into this protective mechanism have been limited to the identification of specific epitopes, usually at a single time point in the infection. Few studies have examined the cellular immune responses to the viral antigens throughout the infection period. Since the virus undergoes rapid mutation following infection, the adaptive immune response must also evolve to meet this challenge. Previously, the EIAV envelope (gp90) protein was shown to be the primary determinant of vaccine efficacy. Here, we hypothesized that the maturation of cellular immune responses is a lengthy process and involves envelope-specific T cell recognition shifting from immunodominant variable determinants to conserved immunorecessive determinants during the initial stages of the EIAV infection. The first part of this dissertation was to develop a new *in vivo* method to identify envelope-specific T cell responses. The second part of this dissertation was to investigate whether envelope-specific T cell recognition evolved in EIAV-infected ponies. Finally, the mechanisms for this T cell immunodominant shifting were also investigated from the point of both virus sequence mutation and T cell clone expansion and contraction. Also, a new EIAV attenuated vaccine which contained a consensus gp90 sequence was tested to see if it facilitated T cell recognition of the more conserved regions early in the infection. Our results indicated that envelope-specific T cell recognition patterns changed over time. Early after infection, dominant immune responses to the peptides in the carboxyl-terminus variable region were identified. By six months post infection, the recognized peptides spanned the entire envelope sequence, with a shift to the amino-terminus conserved region. The mechanisms responsible for this change remain unclear, but analysis of T cell receptor repertoire indicated that T cell clonal expansion and contraction might be one of the reasons. Our demonstration that envelope-specific peptide recognition shifts from the variable to the more conserved regions provides evidence that the maturation of cell mediated immune response is parallelled with long-term control of this infection.

Keywords: Equine infectious anemia virus, Cellular immune responses, immunodominant recognition, immunodominant shifting, consensus sequence

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THE MATURATION OF CELLULAR IMMUNE RESPONSES IN EQUINE INFECTIOUS ANEMIA VIRUS-INFECTED HORSES

BY

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July 28, 2013

Dedication

This doctoral dissertation is dedicated to my beloved family members:

Father: Decai Liu

Mother: Wenxia Ni

Husband: Jinpeng Liu

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Review of the Literature

1.1 EIAV is a model for lentivirus protection

1.1.1 EIAV structure

Belonging to lentivirus genus in the retroviridae family, equine infectious anemia virus (EIAV) shares genetic and structural similarity with HIV, SIV and FIV (Casey et al., 1985; Charman et al., 1976; Montelaro et al., 1988). EIAV is the most genetically simple lentivirus with a genome size of 8.2kb (Leroux et al., 2004). Besides long terminal repeats (LTR) which serve as the site of transcriptional initiation (Carvalho and Derse, 1993; Sherman et al., 1988), the genome contains three major open reading frames, encoding Gag, Pol, and Env proteins. The EIAV *gag* encodes four major internal structural proteins: the matrix (MA) p15, the capsid (CA) p26, the nucleocapsid (NC) p11 and p9 (Hussain et al., 1988; Stephens et al., 1986). These proteins are critical for virus assembly and budding from infected host cells (Parent et al., 1995; Puffer et al., 1997; Strack et al., 2003; Swanstrom and Wills, 1997). EIAV *pol* encodes the enzymatic proteins: RNaseH p66, protease p12, dUTPase p15 and an integrase. The reverse transcriptase (p66/p51 heterdimer) is a proofreading-lacking enzyme and numerous mutations will be generated in the viral genome each time the virus genome is replicated (Preston et al., 1988; Roberts et al., 1988). These mutations provide the virus a molecular basis to evolve rapidly and evade the host immune responses(Goodenow et al., 1989).

EIAV *env* encodes the surface unit (SU) gp90 and transmembrane (TM) gp45 glycoproteins. By non-covalently linkage, gp90 and gp45 form heterdimers. Gp45 is a

transmembrane protein and helps anchor the complex in the lipid bilayer. Gp90 is the surface protein which can bind the ELR1 receptor on equine macrophage and is essential for virus entry (Zhang et al., 2005). Envelope protein sequences, especially gp90, are highly variable *in vivo* (Kim and Casey, 1994). Longitudinal sequencing analysis of viral RNA in plasma from a chronically infected pony revealed that gp90 sequence mutations are not randomly distributed (Leroux et al., 1997). Eight hypervariable regions (V1-V8) were identified and spanned the whole regions of gp90, where most of variable regions were located in the carboxyl terminal of gp90. The variability the gp90 modulates the antigenicity of the gp90 glycoprotein, which is the major target for the immune system. Sequence changes help EIAV escape host immune control (Sponseller et al., 2007). Some studies found that increasingly neutralization-resistant phenotypes emerged during persistent infection (Zheng et al., 1997b). Both gp90 and gp45 are extensively glycosylated, with 18 predicted N-linked glycosylation sites on gp90 and four on gp45. The glycosylation can also help to mask the virus from the immune system. Being the most variable protein in the virus, envelope variation has become a major obstacle in the development of a successful vaccine. Some studies found that even minor changes in envelope sequences will significantly impact the outcome of vaccine efficacy.

In addition to the proteins coded by *gag, pol* and *env*, EIAV has three additional short ORFs: Rev, Tat and S2. The Rev protein of EIAV is functionally similar to other lentiviruses and regulates expression of incompletely spliced mRNAs, multimerizes, and facilitates export of incompletely spliced viral mRNAs (Carpenter and Dobbs, 2010). The EIAV Tat protein shares similar sequence to the other Tat proteins of lentiviruses, but are located predominantly in the cytoplasm in horse (Rosin-Arbesfeld et al., 1994).

Functioning as a transcription activator, Tat protein interacts with the viral LTR and increases the transcriptional activity (Dorn and Derse, 1988). The S2 protein could be detected in the cytoplasm during EIAV infection in horses(Yoon et al., 2000). Some studies showed that S2 may function to organize the Gag protein during particle assembly. Recent studies have indicated that S2 can interact with some proteins which influence the trafficking and degradation of protein complexes in host (Covaleda et al., 2010). Other studies have indicated that S2 is dispensable for viral replication in several equine cell lines(Li et al., 1998), but is a key factor for viral replication and virulence *in vivo* (Li et al., 2000). Based on these evidences, the EIAVΔS2 attenuated strain was developed by insertion of two stop codons into the S2 gene of the infectious molecular clone $EIAV_{UK}$(Li et al., 2003). The insertion of two stop codons did not affect *tat*, *env* or the *rev*-response element. A second deletion form EIAVΔS2 mutant ($EIAV_{D9}$) was developed by insertion of a nine base pair deletion in EIAVΔS2 (Craigo et al., 2007a). Studies have indicated that these attenuated strains could serve as effective vaccines to prevent infection and clinical disease following virulent challenge (Craigo et al., 2007c; Li et al., 2003). Also, these designed attenuated vaccines could serve as models to investigate the mechanisms that correlate with lentiviral protection.

1.1.2 Clinical course of EIAV infection

EIAV is a macrophage-tropic lentivirus which causes persistent infection, characterized in three stages: acute, chronic and inapparent(Issel and Coggins, 1979). Unlike its lentiviral relatives which slowly cause the collapse of the immune system and death of the host due to opportunistic infection, EIAV infection could be effectively

controlled in horses (Clements et al., 1994; Oaks et al., 1998). Thus, one month post experimental infection, most infected horses will develop an acute episode of disease characterized by fever, diarrhea, edema, lethargy, anemia, thrombocytopenia and viremia. During this acute phase, the immune system temporarily controls virus replication and cause a drop of virus load in plasma. Subsequent EIAV escape from the immune response causes another round of rapid viral replication, and clinical symptoms of EIA (Lichtenstein et al., 1996; Sponseller et al., 2007). In the chronic phase, EIAV infected horses show recurrent disease episodes. Sequencing of plasma virus from fever episodes indicated that each occurrence of disease episode is caused by a noval viral quasispecies that is different from the previous episode (Leroux et al., 1997; Zheng et al., 1997a).

After eight to twelve months of chronic disease, most horses will become inapparent carriers free of clinical signs of disease while viral replication is maintained in low levels in monocyte-rich tissue reservoirs. Administration of immunesuppressing drugs can cause the reoccurrence of the disease(Kono et al., 1976). Sequencing of plasma virus indicated that viruses from inapparent carriers were different from the last febrile episode, indicating that EIAV still undergoes variability during inapparent carrier stage although with a low tendency to cause any clinical disease(Leroux et al., 1997). Also, control of the inapparent stage is not attributed to virus attenuation, as whole blood transfers from inapparent carriers will cause disease in naïve horses. In addition, inapparent carriers are resistant to challenge with other viruses, thus demonstrating the development of a high level of protective immunity.

Compared with other virulent lentiviruses, EIAV is unique in that despite aggressive virus replication and rapid antigenic variation, more than 90% of EIAV infected

animals will end up in an inapparent carrier stage, in which the infected animals are free of clinical diseases for the life span of the animal. Thus, the EIAV system has provided a useful model to investigate mechanisms that correlates with protection.

1.2 Host defense mechanisms to viral infections

There is a stratified system of host immunity against invading pathogens. After the physical barriers have been breached, there are two major categories of host immune responses: innate and adaptive immune responses that can be directed against viral infection.

1.2.1 Innate immune response against viral infection

The innate immune response is critical for the initial detection of invading viruses and subsequent activation of the adaptive immune response. The innate immune response recognizes viral components by pattern-recognition receptors (PRRs)(Akira et al., 2006). There are three major classes of PRRs that involved in the recognition of viral components: Toll like receptors (TLRs), RIG like receptors (RLRs) and NOD like receptors (NLRs) (Mogensen, 2009) . These receptors recognize viral components, such as viral genomic DNA, single strand RNA (ssRNA), double strand RNA (dsRNA), RNA with triphosphate ends and viral proteins, and activate signaling pathways leading to the production of proinflammatory cytokines, type I IFNs, and chemokines (Iwasaki and Medzhitov, 2004; Kato et al., 2005). Proinflammatory cytokines and chemokines are important for eliminating virus infection by stimulating inflammation and recruiting innate and acquired immune cells to infection sites. Also, type I IFNs are critical cytokines produced after viral

infections. Type I IFNs are not only produced by professional innate immune cells, such as dendritic cells (DCs) and macrophages, but also by other cells, such as fibroblasts. Type I IFNs bind to a ubiquitous receptor, and activate signaling pathways that trigger the gene expressions of more than 300 IFN-stimulated genes (ISGs)(Randall and Goodbourn, 2008). These effector molecules can directly influence protein synthesis, cell growth and survival and establish an antiviral state. For example, the protein ISG15 prevents virus-mediated degradation of IRF3, enhance NF-κB signaling pathway, and modulate the immune response (Harty et al., 2009). Also, type I IFNs induce maturation of DCs by up regulating gene expression of co-stimulatory molecules such as CD80, CD86, CD40, and class MHC I (Theofilopoulos et al., 2005). In response to certain viral infections, type I IFNs also enhance NK cell cytotoxicity such that a broader range of virus-infected cells are lysed (Yokoyama et al., 2004). Finally, type I IFNs cause stimulation and recruitment of lymphocyte and monocytes at sites of infection by promoting vascular adhesion molecule expression, induction of antigen-specific $CD8^+$ T cell responses and induction of chemokines(Honda et al., 2005).

Natural killer (NK) cells are another important component of innate immunity to eliminate virus-infected cells. NK cells can respond to viral infection rapidly without prior immunization, and are shown to play a critical role in controlling different types of viral infections, such as lymphocytic choriomeningitis virus (Welsh, 1978), paramyxovirus (Anderson et al., 1977), flavivirus (Macfarlan and White, 1980), and herpesvirus. NK cells comprise 5–20% of all lymphocytes in peripheral blood and some specific organs, like the spleen and liver. NK cell inhibition and activation are regulated by the differential engagement of cytokines, inhibitory receptors, activation receptors and corresponding

ligands (Cerwenka and Lanier, 2001; Lanier, 1998). Inhibitory receptors on NK cells recognize MHC class I molecules (Takei et al., 1997; Yokoyama et al., 1995). Normally, inhibitory receptors provide dominant signals over those provided by activation receptors when interacting with healthy cells expressing normal levels of MHC class I molecules(Yokoyama, 1995). However, many types of viruses have developed ways to interfere with the ability of MHC class I molecules to bind and present antigen-specific peptides on the surface of cells (Beck and Barrell, 1988). Thus, NK cells selectively recognize and kill virus-infected cells showing reduced levels of MHC class I molecules. Upon activation, NK cells initiate apoptosis of target cells by granule exocytosis (Smyth and Trapani, 1995; Trapani and Smyth, 2002) or Fas-FasL pathways (Nagata, 1997; Smyth et al., 2003). Also, NK cells can kill infected cells via antibody-dependent cellular cytotoxicity (ADCC) by engagement of their Fc receptors with the Fc portion of antibodies bound to cell-associated antigens(Isitman et al., 2012). Besides cytotoxicity, NK cells can secrete different sets of cytokines and chemokines, such as IFNγ, TNFα, IL-5, IL-10, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory protein (MIP-1α)(Biron et al., 1999; Maghazachi, 2010). These NK cell-released cytokines and chemokines are critical in downstream events of virus infection control and affect the initiation and maintenance of subsequent adaptive immune response(Biron et al., 1999).

Some cells from innate immune responses serve as a bridge between innate and adaptive immunity. Viruses are engulfed by antigen presenting cells (APC), such as dendritic cells (DC) or macrophages(Cella et al., 1997). The pathogens are processed and pathogen-derived peptides are presented on the surface of the APC in the context of MHC.

There are two major classes of MHC molecules: MHC I and MHC II. MHC I molecules bind with peptides (9-10 amino acids) derived from intracellular pathogens, such as viruses, and present to $CD8^+$ T cells. MHC II molecules could bind with peptides (normally 13 amino acids) derived from extracellular pathogens, and present to $CD4^+$ T cells. APC deliver three different signals to naïve T cells for proliferation and differentiation. Recognizing the MHC:peptide complexes on the surface of APC by T cell receptors is the first signal. Signal 2 involves B7 proteins on APCs interacting with CD28 on T-cells. This signal is quite critical for promoting T cell survival and expansion. Also, APCs produce a mix of cytokines in response to different pathogens, and this third signal is primarily involved in directing T cell differentiation into different sets of effector T cells.

1.2.2 Adaptive immune responses against viral infection

Once activated, T cells begin to produce IL2, which is critical for T-cell expansion. Depending on the cytokines received, CD4+ T cells can differentiate into at least four subsets: T_H1, T_H2, T_H17 and Treg. For example, CD4+ T cells differentiation into the T_H1 subset is promoted by IL12 and IFNγ, and differentiation into T_H2 subset is promoted by IL-4. Some recent studies have shown that $CD4^+$ T cells are important components in some viral infections(Sant and McMichael, 2012; Swain et al., 2012). It has been shown that $CD4^+$ T cells can trigger the recruitment of other lymphoid cells into infection sites (Kumamoto et al., 2011). For example, $CD4^+$ T cells can promote recruitment of DCs or antigen-specific effectors to virus replication sites (Beuneu et al., 2006; Castellino et al., 2006). Also, viral infections often induce strong effector CD4+ T cell responses that provide support for expansion or function of B cell and CD8+ T cell responses(Elsaesser,

2009; Wiesel and Oxenius, 2012). Following viral infection, the T_{FH} cells promote the formation of germinal centers, the generation of memory B cells and plasma cells(Crotty, 2011). Interactions between CD40 (B cells) and CD40L (CD4+ T cells) are crucial for the promotion of B cell activation and antibody production. Also, CD4+ T cells are required to generate and enhance CD8+ T cell cytotoxic activity and maintain functions of memory CD8+ T cell populations(Yi et al., 2009). Finally, CD4+ T cells function directly through production of a mix of cytokines or cell-mediated cytotoxicity(Swain et al., 2012). One of the most important cytokines CD4+ T cells secrete is IFNγ. IFNγ mediates a number of different immunoregulatory effects to promote resistance to viral infection(Schroder et al., 2004). It can promote T cell and B cell differentiation, activate CD8+ T cell cytotoxic activity, and enhance the function of macrophages. Also, IFNγ can induce production of a nitric oxide synthase (NOS) enzyme, which triggers the production of reactive oxygen intermediates. These molecules function to modify a variety of molecules important for replication of particular viruses.

Lacking a biosynthetic apparatus, all viruses need to replicate in the cytoplasm of infected cells. Once inside cells, viruses are not accessible to antibodies, and activated $CD8^+$ T cells are in charge of eliminating these infected cells. Naïve $CD8^+$ T cells will differentiate into cytotoxic T cells (CTLs). As mentioned above, the activation of $CD8^+$ T cells also need three signals (interaction between TCR and MHC-peptide complex, co-stimulatory interactions and cytokines). Besides that, the fully activation of $CD8^+$ T cells require more co-stimulatory activity which can be presented in two different ways. The first way is stimulation provided by mature dendritic cells. These cells can stimulate $CD8^+$ T cells to synthesize IL-2 and drive their own proliferation and differentiation. The

second way is extra help provided by CD4 effector T cells. These CD4 effector T cells can trigger dendritic cells to express higher levels of co-stimulatory activity. After activated, CTLs circulate and identify infected cells which present virus peptides on the surface in the context of MHC I molecules. Binding of the T cell receptor (TCR) of CTLs with peptide:MHC I complex on infected cells will trigger the release of lytic granules from CTLs into the synapse between CTLs and the infected cell. The granules contain potent mediators which include pore-forming protein (perforin), cytotoxic cytokines, and granzymes (a family of serine esterases)(Wong and Pamer, 2003). Together these mediators can induce death of the target cells via caspase-dependent and independent apoptotic pathways. Perforin can help granzymes enter infected cells by making pores on the infected cells or help granzymes bind with specific receptors on the surface of infected cells. Once inside infected cells, granzymes will initiate a caspase dependent or independent pathway, and trigger the infected cells to undergo apoptosis (Wiesel et al., 2009). After that, the CTLs disengage with the target cells and move to the next infected target. Additionally, CTLs also release cytokines, such as IFNγ and TNFα. IFNγ can inhibit viral replication in various ways. For example, IFNγ could stimulate the expression of MHC I molecules on the surface of infected cells, which will increase the chance that infected cells are recognized by CTLs. Thus, CTLs act in a variety of ways to inhibit the spread of virus and viral replication in infected cells.

Humoral immune responses also play an important role in controlling virus infections (Sherman et al., 1983). Binding with circulating virus with its receptor (IgM), B cells internalize the virus, process the virus inside cells, and finally present virus-derived peptides on the surface in the context of MHCII molecules. Th cells recognize the MHCII-

peptides complex and activate the B cells. The activated B cells will undergo class switching and secrete virus-specific antibodies. Antibodies have multiple mechanisms to remove viruses from circulation. Neutralizing antibodies are found in many virus infections, and many studies have found a correlation between neutralizing antibodies and protection. Some antibodies can coat the virus and bind the Fc receptors on the surface of phagocytic cells, such as macrophages and neutrophils. The phagocytic cells will phagocytize the virus and degrade viruses in the cells. Also, some antibodies have the ability to activate the complement system, which also can enhance uptake by phagocytic cells.

1.3 Immune responses associated with lentiviral infection

There are two major classes of virus: acute viruses (such as influenza, smallpox, polio and measles) and chronic viruses (such as Epstein-Barr virus, cytomegalovirus, hepatitis virus and HIV-1). Compared with the acute viruses that can be quickly cleared by immune responses, chronic viruses normally establish persistent infection. Although much knowledge has been created in the study of HIV, the exact immune response that is crucial to control HIV infection is still unknown. Studies on other chronic virus and simian immunodeficiency virus (SIV) indicated that both neutralizing antibodies and cell-mediated immunity are effective against viral infection. In this section, we will mainly focus on the immunity that mediates protection in lentiviral infections.

1.3.1 Humoral Immune Responses

A number of studies have investigated the role of humoral immune responses in

controlling lentivial infection(Pantaleo and Koup, 2004). One of the most important components of the humoral immune response against viral infection is neutralizing antibodies, which can neutralize viral particles and prevent viral entry. Although some early studies have found that neutralizing antibodies are present in long-term nonprogressors (LTNPs), later studies have indicated a positive relationship between the levels of HIV neutralizing antibody and plasma virus load. Also, continuous and rapid generation of escape mutants decreases the efficacy of neutralizing antibodies in suppressing viral replication. Thus, it is still unclear what role neutralizing antibodies play in chronic HIV infection. Recent studies have indicated that cross-reactive neutralizing antibodies arise in about 20% of HIV infected patients, and that these cross-reactive neutralizing antibodies mainly target conserved regions and play as a critical role in the protective immune responses to HIV infection (Liao et al., 2013; Mouquet and Nussenzweig, 2013).

In additional to neutralizing antibodies, other types of antibodies also showed effectiveness against lentiviral infection. It has been shown that non-neutralizing antibodies can recruit NK cells and monocytes to mediate a stronger ADCC response (Stratov et al., 2008). Binding to cell-associated antigens, the Fc portion of non-neutralizing antibodies engage with Fc receptors on the surface of innate immune cells, and activate these cells to kill infected targets. In LTNPs, ADCC activity appears to be higher compared to individuals with progressive disease (Alter and Altfeld, 2009).

1.3.2 Cellular mediated immunity (CMI)

One of the major hallmarks of HIV infection is the degeneration of $CD4^+$ T cell

population. Initially, it was thought that strong HIV-specific CD4+ T cell responses may trigger the replication and dissemination of virus. However, recent studies revealed that $CD4^+$ T cells are clearly linked to immune control of HIV. The initial encounter with virus triggers T cell expansion and it has been shown that Gag-specific T cells proliferate better and establish a stronger antiviral activity (McKinnon et al., 2012). These virus-specific $CD4^+$ T cells responses are present in LTNPs. The ability of these virus-specific $CD4^+$ T cells to secrete IL-2 and proliferate upon stimulation is necessary for the preservation of $CD4^+$ T cells function in LTNPs.

Increasing evidence indicates that virus-specific cytotoxic T cell (CTLs) play an important role in controlling lentiviral infection. Some evidence indicates that HIV resistance is associated with specific class I HLA alleles. Since class I HLA alleles are involved in presenting virus-derived peptides to $CD8^+$ T cells, the association between HIV resistance and some specific class I HLA alleles implies that $CD8^+$ T cell responses play a vital role (Heeney et al., 1999). The major antiviral mechanism of $CD8^+$ T cells is to lyze viral infected cells in which perforin and granzymes are major effector molecules. Some studies have indicated that upregulated perforin expression of virus-specific T cell is correlated with protection. Other studies have shown that the ability of CD8 T cells to load lytic granules following antigen encounter is more effective in LTNPs (Betts et al., 2006; Poudrier et al., 2012). Following depletion of $CD8^+$ T cells *in vivo*, macaques had significantly increased viral loads after challenge with SIV. Also, HIV-specific CTLs were detected in long term non-progressers (LTNPs), and there was a correlation between CTLs and low virus load (Betts et al., 2006). However, inconsistent results were found when longitudinal studies were conducted. Additionally, no differences in the magnitude of HIV-

specific T cell responses between persons with chronic progressive disease and LTNPs were detected (Betts et al., 2001).

Indeed, there are multiple examples of detectable virus-specific T cell responses in either infected or vaccinated individuals. However, not all virus-specific T cell responses are necessarily associated with protection against lentiviral infection. Recent research suggests that the quality, but not the quantity of the $CD4^+$ and $CD8^+$ T cell responses may be much more important in protecting against lentivial infections. It has been found that virus-specific T cells maintained a greater ability to produce multiple cytokines (IFNγ, TNFα, MIP-1, CD107a and IL-2) in LTNPs compared to progressors. Also, studies demonstrated that T cells recognizing epitopes (B27-KK10) related with the low viral load were characterized by polyfunctional capabilities, increased clonal turnover, and superior functional avidity.

1.3.3 Antigen sensitivity and TCR avidity

During viral infection, the responding T cells are a heterogeneous population that differ in TCR expression, cytokine production profiles, cytotoxic activity, and homing patterns. Recently, more studies have indicated an important role for antigen sensitivity (AgS) in T cell control of lentiviral infections (Appay and Iglesias, 2011). AgS (also called functional avidity) is the strength of T cell interaction with their targets, and is a composite measurement of multiple interactions between the T cells and targets. It can be determined by a functional assay with decreasing concentrations of antigen, and the AgS for that T-cell response is set at the concentration when 50% of the maximal functional response is achieved (EC50). During acute infection, T cells with high AgS expand much faster than

T cells with low AgS. Also, T cells with high AgS have the capability to produce higher cytokines compared with low AgS T cells, and are more potent in suppressing HIV replication *in vitro.*

There are several factors that influence the AgS, including TCR avidity (affinity of TCR with peptide/MHC complex), interactions between TCR and the CD8 coreceptor, the localization of the TCRs, the accessory molecules in lipid rafts, and the microenvironment (cytokines)(Stone et al., 2009). TCR avidity is one of the most important factors that influence AgS and efficacy of T cells against lentiviral infection (Bennett et al., 2010). For situations such as HIV infection, where epitope variability is high, the T cell response needs to adapt to the new variant or to be more cross-reactive, thus a greater breadth of TCRs may be better for immune containment. The diversity of TCR is generated in the process of combinatorial associations of germline V(D)J regions. The numbers of TCRs recognizing one epitope may be more than one, and the clonotypes of TCR against the same epitope may be highly variable between individuals. The TCR repertoire influences its qualitative attributes. It has been found that different TCR clonotypes influence the control of HIV-1 replication *in vitro* via cross-reactivity to epitope variants and enhance ability to load and deliver perforin.

Although much evidence supports an important role of AgS and TCR avidity in lentivial infection, there are divergent opinions as well. Firstly, due to the strong selection pressure that T cells (high AgS) exert on the virus, the virus may mutate and escape from the immune control. Second, T cells with high AgS rapidly proliferate after viral stimulation, which may drive them towards T cells exhaustion and irreversible clonal depletion. Lastly, not all evidence supports a positive correlation between the AgS and

viral control. It has been found that some protective polyfunctional T cells actually have low avidity for some HIV epitopes. In fact, these diverging data might indicate a multifaceted basis of AgS.

In summary, although there are many promising data which indicate the importance of CMI responses in controlling lentriviral infections, the key attributes of T cell mediated protective immunity in lentiviral infection remain unclear. Considering the profound heterogeneity and complication of T cells responses, assays to accurately identify those responses are needed.

1.4 Questions regarding assays to measure CMI responses in lentivirus systems

Lots of data have shown that CMI is important in controlling lentiviral infections, but precise parameters of CMI that correlate with protection are still unknown. One possible interpretation is that the method used to measure CMI responses is questionable (D'Souza and Altfeld, 2008). There are several ways to measure CMI responses: tetramer-staining assays can be used to determine the frequency of virus-specific T cells using common MHC-restricted epitopes(Lieberman, 2004). Another commonly used assay is to measure the IFNγ production by ELISPOT assay or intracellular cytokine staining (ICS). ELISPOT has been widely used in both basic and clinical research to detect CMI responses in HIV infected patients(Lieberman, 2004). Similarly, as in the tetramer-staining assay, ELISPOT assays detect the frequency of IFNγ producing cells. However, recent studies have started to call into question the reliability of ELISPOT assays for the measurement of effective CMI responses. It has been found that though virus-specific T cells maintained

high frequencies until late in disease, many of them have altered functional abilities (Richmond et al., 2011), which both tetramer-staining and ELISPOT assays fail to detect. Also, usage of only one functional indicator (IFNγ) may miss other effector responses. Although IFNγ has antiviral effects, perforin and granzymes are more direct effector molecules killing infected cells (Kuerten et al., 2008). Additionally, due to the low frequency of antigen-specific T cell responses detected, it is very easy to generate false positive, especially by unblinded researchers.

In addition to the limitations of methods used to detect CMI responses, there are other issues of concern in lentiviral research field. The first concerns using consensus viral sequences to construct antigens. Considering that the virus continuously mutates in *vivo*, this method can only be an approximate measurement. Also, T cells recognizing earlier virus may have better functional properties than the T cells recognizing later virus variants. Indeed, some studies suggested that the use of consensus viral sequences underestimates some T cells responses, and its breadth (Altfeld et al., 2003). Another problem is that some studies investigating protective immunity are often cross-sectional. It has been found that CMI responses sometimes are low and intermittent, which means sampling at a single time point may underestimate transient responses.

Considering these methodological weaknesses, it is time to think about alternative approaches. Choosing the right assays is especially important for investigating the functional activity of T cells following lentiviral infections. Also, improved assays can provide powerful tools for studying the protective mechanisms and further the development of an effective vaccine against HIV.

1.5 Immunodominance and immunodominance shifting

1.5.1 Immunodominance and the mechanisms of immunodominant shifting

With complex viral antigens, not all potential epitopes can be recognized by CD4 or CD8 T cells equally. Also, not all recognized epitopes elicit equivalent responses (Yewdell and Bennink, 1999b). Normally, T cells focus on one or a few epitopes (immunodominant epitopes). The rest of epitopes (subdominant epitopes) can still elicit T cells responses, but at lower levels. During the acute virus infection, immune responses function rapidly and eradicate the infection. At the same time, a pool of memory T cells remain stable following viral clearance and confer long-lived immunological protection. In the case of the chronic virus infection, however, most viruses can not be controlled rapidly by the host's immune responses. In this situation, a shift of immunodominance is frequently detected evidenced by the changes in specificity, breath and magnitude of epitopes-specific responses. In addition, the composition of the T cells pools may change over time (Fuller et al., 2004; Wherry et al., 2003).

There are two major parameters that may shape the immunodominance: antigen-related factors and T cell-related factors (Yewdell and Bennink, 1999a). Antigen processing inside APCs may be the primary filter for epitope selection. After entry, virus proteins will be degradated by the immunoproteasome, then the processed peptides will be transported to the endoplasmic reticulum, bind with MHC molecules and finally translocate to the cell surface. Any of steps involved antigen processing and final presentation to the MHC complex can influence immunodominance. Also other factors, such as the kinetics of viral protein synthesis, the overall abundance of the peptides, and the affinity and stability of peptide/MHC complex can influence the hierarchy of T cell responses, as well

(Yewdell and Bennink, 1999a). Additionally, in persistent viral infections, like HIV, where antigens change overtime, immunodominance is largely influenced by the sequence and availability of the peptide antigens. Mutations in the anchoring residues may significantly reduce the affinity and stability of peptides binding with the MHC molecule. In this situation, the presentation of the mutated peptide antigens to T cells may be diminished or out-competed by other subdominant peptides.

For the T cell-related factors, it appears that the T-cell repertoire in the host can shape the immunodominance as well(Gallimore et al., 1998a). During T cell development, thymic selection shapes the diversity of the naïve CD8 T cell repertoire, and influences the antigen-specific T cell precursor frequencies. In order to elicit T cell responses, a threshold number of antigen-specific T cells with a certain affinity within the T cell repertoire is required, thus the repertoire and composition of the pool of T cells are quite important in shaping the initial response and subsequent memory pools. In chronic viral infection, studies found that T cell may undergo clone exhaustion during persistent infections. The antigen-specific T cells may succumb to deletion, leading to a change in these T cell repertoire and epitope hierarchies.

It has been found that the initial immunodominant effector pools usually contribute to the structuring of both primary response and the memory pools. Thus factors shaping immunodominance might also influence viral loads, chronicity and outcome of infections. It is thus a concern that different functions between epitope-specific responses exist. When designing vaccines against chronic viruses, people should be aware of the kinetics changes of epitope specific T cells responses, and which immune responses are the most effective at mediating rapid control of primary infections as well as which responses are

subsequently capable of conferring long-lived immunity.

1.5.2 Immunodominance and immunodominant shifting in lentivirus infection

The most conclusive evidence to show the association between T cell specificity and virus control comes from the study of persons with one specific class I HLA allele B*27. It was determined that individuals with class I HLA allele B*27 consistently demonstrate delayed disease progression. Targeting of a single B*27-restricted Gag epitope (KK10) was associated with lower viral load. However, the magnitude of Gag-specific T cell responses is actually lower in LTNPs than in persons with progressive infection. Also, attempts to associate specific epitope responses with lentiviral control on a population level fail to reveal a clear linkage. More studies have supported that protective T cell epitope recognition should focus on conserved areas of the viral genome as T cell responses were shown to be skewed towards variable peptides in early HIV-1 infection, whereas towards more conserved peptides in chronic infection (Bansal et al., 2005; Goulder et al., 2001; Liu et al., 2011c). Other studies have shown that HIV-specific T cells responses could exert selection pressure and cause escape mutations rapidly in the primary infection, but the virus escaped more slowly or was invariant in chronic infections (Goonetilleke et al., 2009). These results indicated that immunodominant epitopes recognized by T cells in the acute phase may serve as immunodecoys, whereas immunorecessive epitopes may be more related to protection (Frahm et al., 2006a; van der Most et al., 1997). HIV infection studies found that the frequency of naive T cell precursors influenced the immunodominant recognition during acute infection. Also, antigen sensitivity and TCR avidity can determine the immunodominance of HIV-specific T cells.

Thus, when designing vaccines, it is important to know whether T cell recognition differs between acute and chronic infection. If so, it is vital to determine which responses should be generated and the appropriate antigens incorporated within a candidate vaccine.

1.6 Host immune response to EIAV infection

Although intensely studied, no effective vaccine against lentiviral infections is available. In contrast to other lentivirus, horses gain immunologic control of the mutating virus offering the possibility to study the protective mechanisms. The importance of the immune response in controlling EIAV infection is supported by several pieces of evidence: administration of immune suppressive drugs to inapparent carriers leads to the recrudescence of EIA (Craigo et al., 2007a). Also, foals with severe combined immunodeficiency (SCID) fail to control the initial viremic episode, and adoptive transfer of EIAV-stimulated lymphocytes restores the control of viral replication in SCID foals. Several studies have suggested that both humoral and cellular immune responses are needed to control EIAV infection.

1.6.1 Humoral immune responses against EIAV infection

After being infected with EIAV for about three weeks, most horses typically become seropositive(Perryman et al., 1988). The humoral immune responses are predominantly focused on the viral envelope glycoproteins, and the antibody titer against envelope is at least 10 fold greater than the antibody titer against the p26 protein. In EIAV infected horses, neutralizing antibodies were originally thought to develop two months post infection, but a recent study has identified neutralizing antibodies in the acute episode of

EIAV infection (Mealey et al., 2005b). Neutralizing antibodies exert an immune selection pressure on EIAV(Rwambo et al., 1990). Each disease episode during the chronic phase of EIAV infection is associated with the emergence of a new neutralization-escape quasispecies. Additionally, sequential EIAV variants that arise during persistent infection showed increasing resistance to neutralization, which indicated that neutralizing antibodies is one of the driving forces for immune selection (Howe et al., 2002). However, contrary results about the role of neutralizing antibodies in controlling EIAV infection are also reported. The level of neutralizing antibodies fluctuates markedly during the course of infection without reaching a stable state. Also, a lack of correlation was detected between the level of neutralizing antibodies and protection in some studies of vaccinated or infected horses(Montelaro et al., 1998b). Facing virus evolution, immune responses may need time to adapt (Hammond et al., 1997; Montelaro et al., 1998a). Hammond et al. described a gradual evolution of the humoral response during the progression from chronic disease to inapparent stages, and found that EIAV-specific antibody gradually matured from low avidity, non-neutralizing, mainly focused on linear epitopes to high avidity, neutralizing activity, mainly focused on conformational epitope (Hammond et al., 1997; Montelaro et al., 1998a).

1.6.2 Cellular mediated immunity (CMI) against EIAV infection

The clearance of the primary viremia correlates with the appearance of EIAV-specific CD8+ T cells (McGuire et al., 1994a). Also, virus mutants escaping from EIAV-Gag and Envelope specific T cells are found (Mealey et al., 2003b). Cellular immune responses against various EIAV proteins, such as envelope gp90 and gp45, the major core

protein p26, can be detected (Mealey et al., 2005a). Also, T cell epitope mapping within Gag matrix (p15), Capsid (p26), Pol, Envelope and Rev have been conducted (Chung et al., 2005; Mealey et al., 2003b). However, vaccines designed to trigger Gag and Pol epitope-specific immune responses are not effective at preventing infection or disease. A true correlate of protection has not been found in EIAV infection. Lentiviruses have one of the most rapidly evolving genomes. Both low fidelity of the lentiviral reverse transcriptase and recombination of individual viral genomes within coinfected cells contribute to the highly mutation rate. Comprehensive studies of EIAV genomic variation in experimentally infected horses revealed that sequence changes predominantly occur in the envelope glycoprotein. Detailed analyses of the evolution of EIAV indicated that each disease episode correlates with the emergence of antigenic variants of the envelope in new predominant quasispecies. A lot of studies have shown that the viral envelope protein has a tremendous impact on the host immune responses to control viral infection and that the mutations observed in envelope quasispecies represents a major challenge to lentiviral vaccine development. Tagmyer has examined in detail the Th and CTL epitopes of EIAV envelope protein in horses vaccinated with the live attenuated strain ($EIAV_{D9}$) of EIAV and found that six months post vaccination gp90-specific CTL and Th cells could recognize peptides spanning the whole region of gp90, which may indicated a mature cellular immune responses against gp90 (Tagmyer et al., 2008a; Tagmyer et al., 2007). No one has studied how CMI evolves into this maturation status. A gradual evolution of the humoral response during the progression from chronic disease to inapparent stages is detected, indicating the maturation of the CMI response might also be a lengthy process.

CHAPTER 2

Hypothesis and specific aims

The lack of a successful HIV vaccine accentuates the need to identify the protective mechanisms involved in lentivirus infections. Equine infectious anemia virus (EIAV) has been used as a model to investigate the protective immunity against lentivirus. Unlike HIV, EIAV replication can be eventually controlled by the host's immune systems, however, the immune mechanism that correlates this protection has not been identified. While the evolution of envelope-specific antibody responses following antigenic variation in the envelope proteins have been investigated in great detail, little information is available regarding the maturation of T cell responses to this virus. The existence of an effective T cell response could exert selection pressure on the virus, forcing mutations to escape this immune pressure. This would result in the disappearance of immunodominant T cell antigens driving the T cells to recognize immunorecessive epitopes representing more conserved amino acid sequences. We hypothesize that the envelope-specific recognition will shift from immunodominant variable determinants to conserved immunorecessive envelope determinants following the virus challenge.

Specific aim one: To develop a new *in vivo* method to identify envelope specific T cell responses in EIAV infected ponies. Lots of data have shown that celluar immunity is important in controlling lentiviral infections, but the true parameter that correlated with protection is still unknown. One possible interpretation is that the methods we used to measure celluar immunity are questionable. Characterization of cellular immunity to lentivirus infection is usually done through the use of *in vitro* assays using peripheral blood

mononuclear cells (PBMC). Considering that less than 2% of lymphocytes reside in blood, PBMC responses may not fully reflect overall cellular immunity. Therefore, we developed a skin test to monitor the EIAV envelope specific cellular immunity *in vivo*. This newly developed method can be used to demonstrate EIAV peptide-specific immune responses *in vivo*.

Specific aim two: To investigate the kinetics of envelope-specific T cell responses in EIAV newly infected ponies. The ability of these lentiviruses to evolve, mainly in its envelope protein, is the primary mechanism of lentivirus persistence To face this high propensity for antigenic variation, we believe that immune responses also evolve. In order to fully understand the kinetics of cellular immune responses, we synthesized forty-four peptides, spanning the entire surface unit protein (gp90) of EIAV, and monitored the peptide-specific T cells responses *in vivo* over six months following infection. This result will provide an insight into the evolution of cellular immunity in EIAV infected horses, that this shift in epitope recognition may associate with the control of viral replication and the establishment of the inapparent carrier state.

Specific aim three: To determine the relationship between EIAV gp90 specific T cells recognition pattern and epitope variability in EIAV newly infected ponies. In persistent viral infections, especially like HIV in which antigens change overtime, immunodominance is largely influenced by the sequence and availability of peptide antigens. Mutations in the anchoring residues may significantly reduce the affinity and stability of peptides binding with the MHC molecule. In this situation, the presentation of

the mutated peptide antigens to T cells may be diminished or out-competed by other subdominant peptides. In this study, the variability of EIAV gp90 in the attenuated $EIAV_{D9}$ stain will be analyzed, and the epitope variability detected at 1, 3 and 6 months post EIAV infection will be compared. This result will provide an insight of EIAV gp90 variation on immune recognition, which may have profound effects on vaccine efficacy.

CHAPTER 3

The determination of *in vivo* envelope-specific cell-mediated immune responses in Equine Infectious Anemia Virus-infected ponies.

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3.1 Summary

Distinct from human lentivirus infection, equine infectious anemia virus (EIAV)-infected horses will eventually enter an inapparent carrier state in which virus replication is apparently controlled by adaptive immune responses. Although recrudescence of disease can occur after immune suppression, the actual immune correlate associated with protection has yet to be determined. Therefore, EIAV provides a model for investigating immune-mediated protective mechanisms against lentivirus infection. Here, we have developed a method to monitor EIAV-envelope specific cellular immunity *in vivo*. Forty-four 20-mer peptides, representing the entire surface unit protein (gp90) of EIAV, were combined into 14 peptide pools and intradermally injected into the neck of EIAV-infected horses. An identical volume of saline alone was injected into a fifteenth site as a negative control. After 48h, those sites with palpable infiltrations were measured prior to the collection of 2mm and 4mm punch biopsies. Total RNA was extracted from each 2mm biopsy for determination of CD3 plus interferon-γ (IFN-γ) mRNA expression by real-time PCR. The 4mm skin biopsies were formalin-fixed and paraffin-embedded for immunohistochemistry (IHC) staining for CD3, CD20, CD25 and MAC387 (macrophage marker). Peripheral blood mononuclear cells (PBMC) were obtained prior to the injection and tested for *in vitro* reactivity against the same peptides. Histological examination showed that some of the envelope peptides elicited a lymphocytic cellular infiltration at the injection site, as evidenced by positive staining for CD3. Peptide-specific increases in CD3 and IFN-γ gene expression were also detected in the injection sites. Furthermore, differences were found between *in vivo* and *in vitro* responses to specific peptides. These

results demonstrate a novel method for detecting *in vivo* cell-mediated immune responses to EIAV-specific peptides that is readily applicable to other host/pathogen systems.

Keywords: EIAV; lentivirus; cellular immune responses; *in vivo*; envelope; DTH

3.2 Introduction

In contrast to lentivirus infections in humans, equine infectious anemia virus (EIAV) replication is eventually controlled in most infected horses (Leroux et al., 2004). Following initial infection, equids may exhibit recurring febrile viremic episodes associated with high viral loads. After 12 to 24 months the frequency of disease episodes begins to diminish and eventually the infected animal becomes free of overt clinical signs and enters an inapparent carrier state that may last for many years (Craigo and Montelaro, 2010). Maintenance of this carrier state is dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce virus replication and the recurrence of disease (Craigo et al., 2002b; Tumas et al., 1994). Inapparent carriers are resistant to re-infection by other strains of EIAV, indicating that they have acquired a certain degree of cross-reactive protective immunity (Montelaro et al., 1993). Therefore the EIAV/horse system provides an opportunity for investigating mechanisms of protective immunity against lentiviruses.

Both humoral and cellular immune responses appear to play important roles in controlling EIAV infection. Virus-specific cytotoxic T cells (CTL) and neutralizing antibodies are detected after the resolution of the acute phase of infection (Leroux et al., 1997; McGuire et al., 2004). There is a progressive maturation of envelope-specific antibody responses in EIAV infected horses, as characterized by the continuing increase in titer, avidity and breadth of epitope reactivity throughout the first year of infection (Hammond et al., 1997). However, little is known regarding the evolution of cellular immune responses during EIAV infectoin. Until this study, characterization of cellular immunity to EIAV infection has been investigated using *in vitro* assays (Chung et al., 2004;

Mealey et al., 2005b). However, these *in vitro* assays may not detect alterations in cellular immune responses occurring *in vivo*. While methods have been developed to monitor *in vivo* immune responses in mice (Ashbridge et al., 1992; Nishino et al., 1994), guinea pigs (Estrada et al., 1992; Mackall et al., 1993) and humans (Sitz et al., 1997a); similar approaches have not been used to monitor cellular immune responses in horses. Here, we have developed a method capable of defining epitope-specific cell-mediated immune responses in EIAV-infected horses. Using this approach, specific differences in epitope recognition between acutely infected and inapparent carrier were identified. There were also differences between *in vivo* with *in vitro* responses to the peptides.

3.3 Materials and method

3.3.1 Animals, virus and experimental challenges

The overall methodology to evaluate CMI responses *in vivo* was developed and optimized using a long-term, EIA inapparent carrier horse (D64) along with four ponies (H40, H41, H42 and H43) experimentally infected within six months of the commencement of these studies. D64 had been infected with the pathogenic viral strain $EIAV_{PV}$ (Rwambo et al., 1990) and had not experienced a febrile episode for more than 7 years. All four ponies (H40, H41, H42 and H43) had the same sire and were challenged with EIAV viruses (EV0, EV6 and EV13) derived from infectious molecular clones of $EIAV_{PV}$ (Craigo et al., 2007d) . A non-infected pony (F31) was used as a negative control. All animals were handled under the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture, according to protocols approved by the University of Kentucky Institutional Animal Care and Use Committee.

3.3.2 Production of synthetic peptides and construction of peptide pools matrix

Initially, forty-four peptides of 20 amino acids in length, overlapping sequential peptides by 10 amino acids and spanning the entire surface unit protein (gp90) of $EIAV_{PV,}$ were synthesized (GenScript USA Inc.,Piscataway, NJ, USA) and used to construct peptide pools 1-7 and A-G as shown in Figure 3.1A. An additional 17 and 26 peptides specific for EV6 and EV13, respectively, were synthesized for the construction of a new peptide pool matrix for testing in the four infected ponies. All the peptides sequences were previously published (Tagmyer et al., 2008b) and each peptide was HPLC-purified, and the purity was confirmed by mass spectrometry (Tagmyer et al., 2007).

All peptides were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) at a stock concentration of 2 mg/ml. Later, the peptides were diluted in saline for determination of the peptide specific responses *in vivo*. The optimal working concentration for individual peptides was determined by serially diluting a known positive peptide (#38) and injecting each dilution intradermally into the long-term infected inapparent carrier (D64) at final concentration of 4.5, 1.5, 0.5, 0.17 and 0.06µg in 100 µl saline. The positive peptide elicited responses at doses greater than 0.17µg (data not shown). In contrast, there was no significant gene expression in response to the negative peptide, #2 at the highest dose tested. Based on these preliminary results, each peptide was tested at a dose of 0.5µg both individually and when incorporated into the peptide pools.

3.3.3 Punch biopsy and sample processing

The neck of each horse was clipped and cleaned with 75% ethanol before intradermal injection of 0.1ml peptide pools or single peptides. Saline alone was injected to serve as the negative control. All injection sites were marked with an indelible marker for identification. After 48hrs, the palpable infiltrations were measured at each injection site and a 2mm skin biopsy was collected and stored in RNALater® (Ambion, Austin, TX) in -20^{o}C. A 4 mm skin biopsy sample was also collected and placed in 10% formalin for subsequent paraffin embedding. The biopsy sites were closed using a single suture.

3.3.4 Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) staining.

Serial 6μm sections were cut from 4mm skin biopsy samples using Leica RM2235 Microtome and placed onto poly-L-lysine-coated glass slides for hematoxylin and eosin (H&E) as well as immunohistochemistry (IHC) staining. H&E staining was performed according to established protocols. IHC staining was performed using automated staining system (BondMAX, Leica Biosystems, Buffalo Grove, IL). The paraffin embedded biopsies were de-paraffinated in xylene, and rehydrated with decreasing alcohol rinses, followed by heat epitope retrieval. An EDTA based buffer and surfactant (pH8.9-9.1) was used for equine CD3, CD25 and MAC387 epitope retrieval. A citrate-based buffer and surfactant (pH5.9-6.1) was used for equine CD20 epitope retrieval. Pan T lymphocytes were detected using a cross-reactive, primary mouse anti-human CD3 antibody (Clone: LN10) and activated T cells with a monoclonal anti-human CD25 antibody (Clone: 4C9). B cells were detected using a primary mouse anti-human CD20 antibody (Clone: 7D1). Neutrophils, monocytes, and macrophages were recognized by primary mouse anti-

macrophage marker antibody (Clone: MAC387). Following primary staining, slides were incubated with a post primary blocking reagent (8mins), horse-radish peroxidase-labeled polymer (8mins) and DAB substrate (10mins). After counterstaining with hematoxylin, sections were dehydrated by increasing graded alcohols and mounted with cover slips. Between additions of each reagent, washing steps were performed using Bond Wash Solutions. All antibodies and reagents were purchased from Leica Biosystems (Buffalo Grove, IL).

3.3.5 Relative quantitation of gene expressions by real-time PCR

The 2mm skin biopsy was transferred in 1.5ml eppendorf tube containing 0.5ml RNA-STAT 60 (Tel-Test) and one 3 mm zirconium oxide bead (Retsch, Newtown, PA). The biopsies were homogenized using a Retsch MM301 Ball Mill (Newtown, PA). All samples were then placed on ice for 30 seconds before a second homogenization. Afterwards, the suspended cell-lysates were transferred to another eppendorf tube with an additional 0.5ml RNA-STAT 60 and stored at -20 ℃ for later RNA extraction. Total RNA was isolated using the phenol: chloroform method (Chomczynski and Sacchi, 1987a). One µg of RNA was dissolved in 41.5µl nuclease-free water (Qiagen, Valencia, CA) and reverse transcribed into cDNA, as previously described (Breathnach et al., 2006) and stored at -20 ℃ until later use.

Equine-specific, intron-spanning TaqMan primers and probes were used for RT-PCR amplification. PCR reactions were performed as previously described (Liu et al., 2011a). Amplification efficiencies were determined using Linreg (Ramakers et al., 2003) and only samples with amplification efficiencies above 99% were included for further

analyses. Beta-glucuronidase (β-GUS) was used as housekeeping gene and the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001a) was used to determine relative gene expression with saline injection site for each horse used as the calibrator. Relative quantity (RQ) was calculated as $2^{-\Delta\Delta CT}$.

3.3.6 Stimulation and measurement of effector cells *in vitro*

Heparinized blood was collected prior to intradermal injections and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) gradient centrifugation according to the manufacturer's protocol. The PBMC (3×10^6) were pulsed with 20ug/ml peptide pools for 4hrs, then transferred to fresh RPMI-1640 (Gibco, Grand Island, NY) supplemented with 2.5% (v/v) fetal equine serum (FES, Sigma, St. Louis, MO), 2mM glutamine (Sigma), 100U/ml penicillin/streptomycin (Sigma), and 55 µM 2-mercaptoethanol (GIBCO, Grand Island, NY). The peptide-stimulated PBMC were incubated *in vitro* for 4 days in a 24 well plate at 37 ℃ in a 5% CO2 incubator. The cells were then pelleted and re-suspended in 1ml of RNA-STAT 60 (Tel-Test) for further analysis of gene expressions by real-time PCR, as described above.

3.4. Results

3.4.1 Identification of peptide responses in an EIAV inapparent carrier

Seven of the 14 peptide pools produced measureable swelling at the injection site in the inapparent carrier, D64, at 48hrs post injection (Figure 3.1B). Furthermore, in biopsy samples from all 7 of these injection sites there were significant increases in expression of CD3 mRNA (Figure 3.1C). It was predicted based on the peptide pool matrix that D64

could respond to a maximum of 12 individual peptides, as indicated by the grey boxes (Figure 3.1B & C). However, subsequent experiments demonstrated these responses were restricted to five peptides (4, 12, 37, 38, and 40) (Figure 3.2A). When these five sites were biopsied and processed to determine CD3 gene expression by real-time PCR, all five injection sites exhibited increased CD3 gene expression (Figure 3.2B). In contrast, there was no evidence of increased CD3 gene expression at sites injected with peptides that produced no measurable lymphocyte infiltration (data not shown).

A

	A	B	C	D	E	F	G
1	1	3	5	7	9	11	13
2	15	17	19	21	23	25	27
3	29	31	33	35	37	39	41
4	43	2	4	6	8	10	12
5	14	16	18	20	22	24	26
6	28	30	32	34	36	38	40
7	42	44					

B

	A	B	C	D	E	F	G	
1	1	3	5	7	9	11	13	0
2	15	17	19	21	23	25	27	0
3	29	31	33	35	37	39	41	49
4	43	2	4	6	8	10	12	500
5	14	16	18	20	22	24	26	0
6	28	30	32	34	36	38	40	625
7	42	44						0
	0	0	150	0	100	150	300	

Areas of palpable infiltrations(mm2)

Areas of palpable infiltrations(mm2)

C

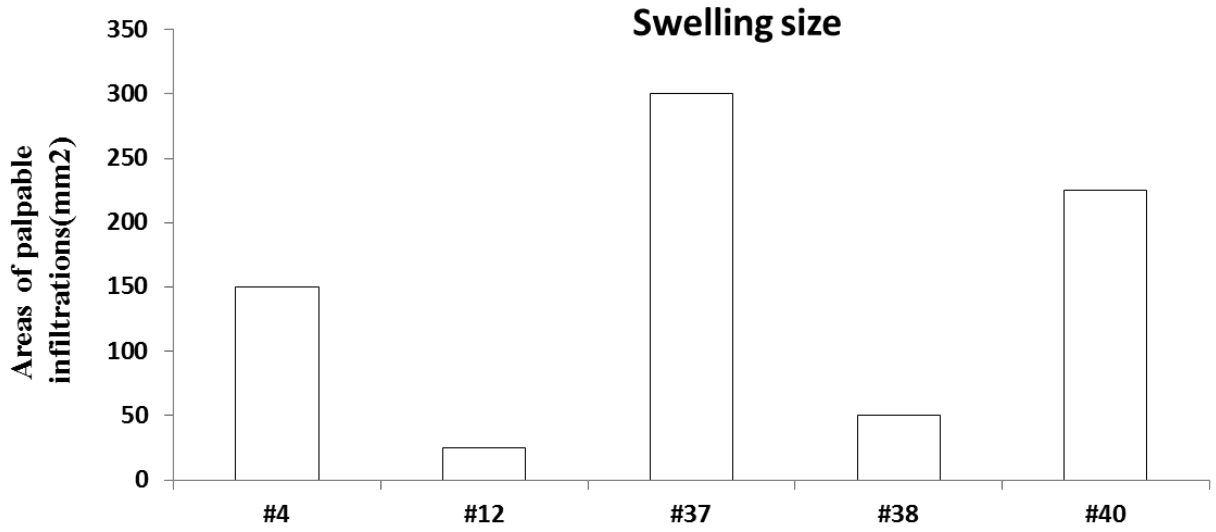
	A	B	C	D	E	F	G	
1	1	3	5	7	9	11	13	1.47
2	15	17	19	21	23	25	27	1.05
3	29	31	33	35	37	39	41	2.37
4	43	2	4	6	8	10	12	4.26
5	14	16	18	20	22	24	26	1.67
6	28	30	32	34	36	38	40	3.28
7	42	44						0.93
	0.80	1.70	3.12	1.33	4.76	2.40	2.33	

RQ of CD3

RQ of CD3

Figure.3.1 Identification of single peptide responses by peptide pool screening. (A) The peptide components of each pool used for injection. (B) Predicted single peptide reactivity as determined by the physical response and (C) CD3 gene expression for each pool. Intersecting pools (gray shading) identify possible peptide specificities.

A



B

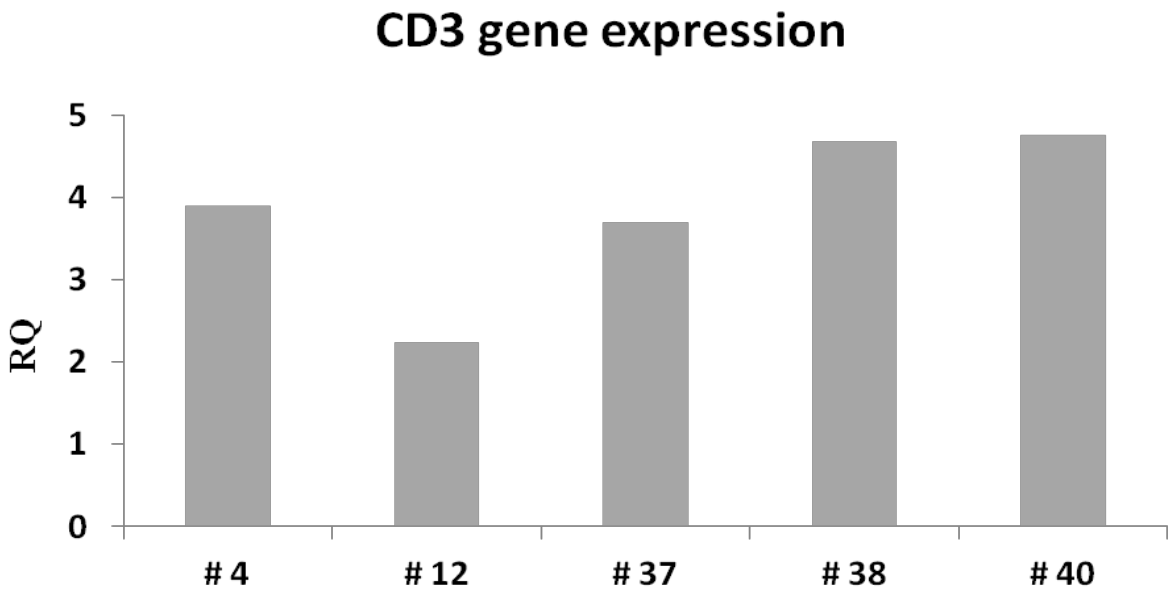


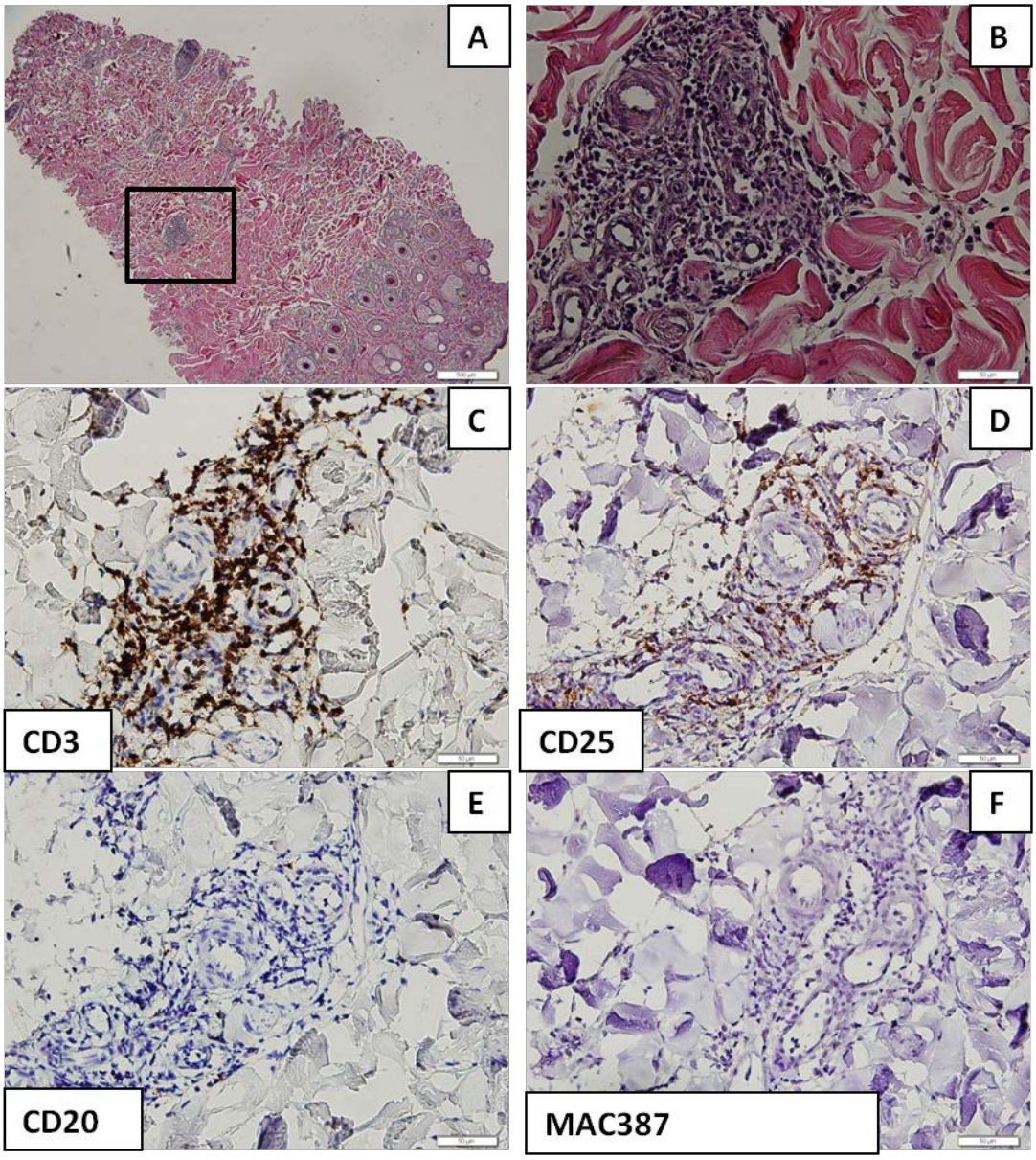
Figure.3.2 Confirmation of individual peptide reactivity in D64. Single peptides 4, 12, 37, 38 and 40 were injected into the neck and physical responses measured after 48 hours. Positive responses were indicated by measurable palpable infiltrations (A) and increased CD3 gene expression (B).

3.4.2 Histopathology in response to injection with an individual reactive EIAV gp90 derived peptide.

Biopsy samples from saline and a positive peptide (#37) injection site were selected for histopathologic examination. H&E staining showed that injections of the peptide induced vasodilation and recruitment of lymphocytes to the injections site (Figure 3.3A & B). The recruited cells were mainly activated T lymphocytes as determined by CD3 and CD25 staining (Figure 3.3C & D). There were few B cells or macrophages present (Figure 3.3E & F). No vasodilation or cell recruitment occurred in the sites where saline was injected (Figure 3.3G & H). Likewise, CD3 and CD25 staining showed only resting T cells randomly dispersed (Figure 3.3I & J) and few B cells or macrophages detected (Figure 3.3K & L) in the saline injection site. Similar results were seen for the other positive peptides (data not shown).

3.4.3 *In vitro* and *in vivo* IFNγ gene expression following stimulation with EIAVgp90 derived peptide pools

Following intradermal injection of D64 with EIAVgp90 derived peptide pools IFNγ gene expression was only significantly up-regulated within those sites receiving pools C, E, F, G, 3, 4 and 6 (Figure 3.4). Therefore, as predicted the results closely mirrored those obtained during transcriptional analysis of CD3. Stimulation of PBMC prepared from D64 with the same peptide pools produced increases in IFNγ gene expression in cells receiving pools E, F, G, 3 and 6 as found *in vivo*. However, in contrast to the *in vivo* results there was no increases in IFNγ gene expression to pools C and 4, although transcription of this gene was up-regulated following the addition of pools A, 1 and 7 (Figure 3.4).


A
B
C
CD3
D
CD25
E
CD20
F
MAC387

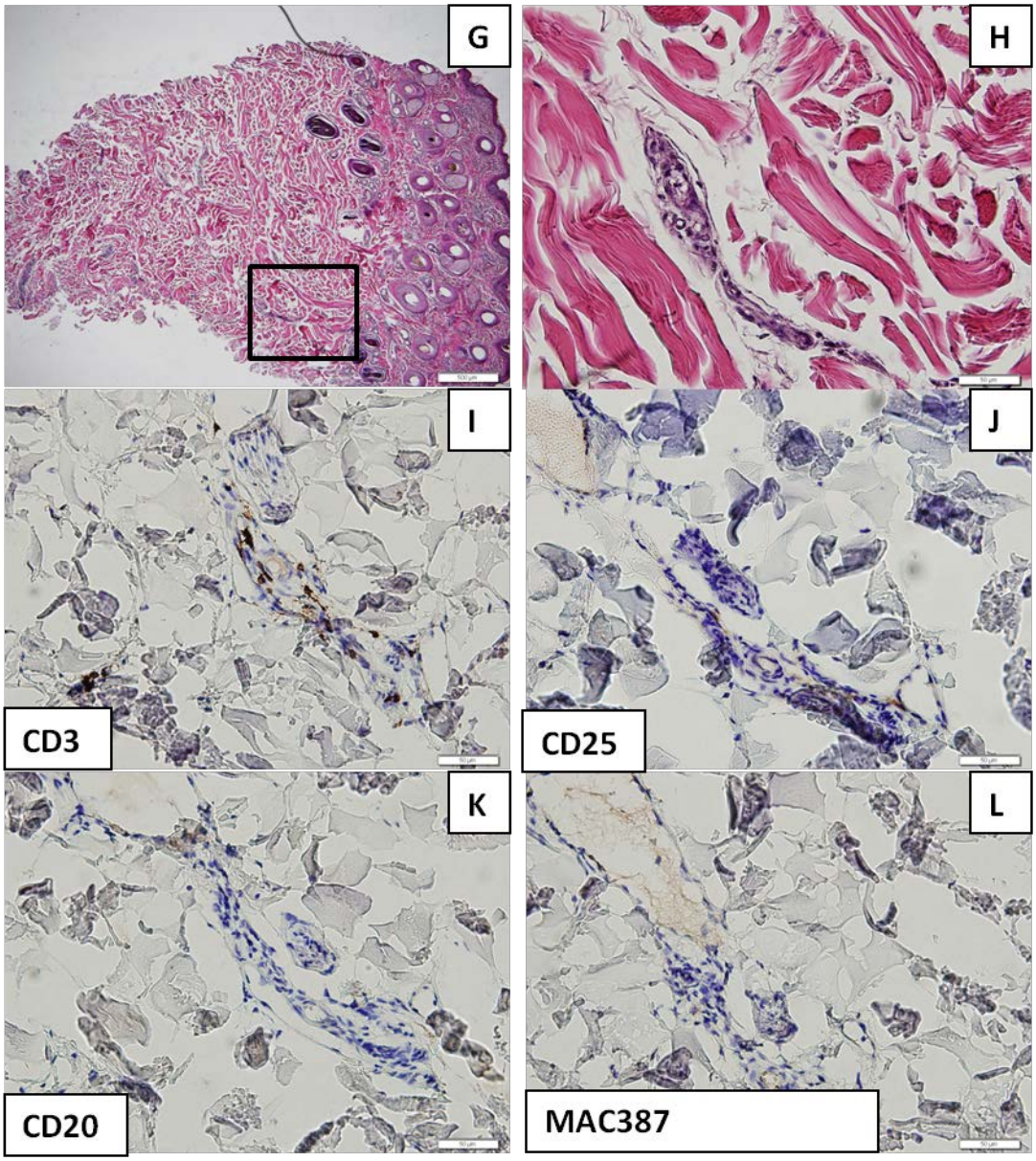
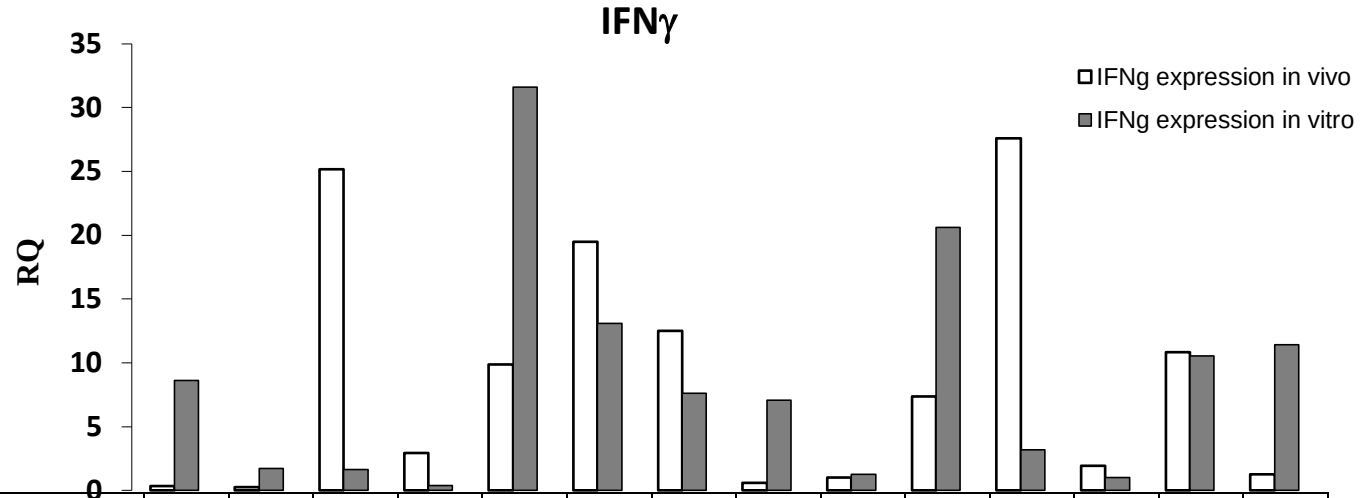

G
H
I
CD3
J
CD25
K
CD20
L
MAC387

Figure.3.3 Histopathology of #37 peptide injection site from D64. Sections (A-F) and saline alone (G-L) ware stained with H&E (A,B,G,H) or with CD3 (C,I), CD25 (D, J), CD20 (E, K) or MAC387 specific antibodies (F, L). Images (A and G) are shown at 4X magnification and the boxed area represents area used for subsequent imaging at 40 X for each of the antibody stains.



Peptide pools	A	B	C	D	E	F	G	1	2	3	4	5	6	7
IFNγ expression *in vivo*			x		x	x	x			x	x		x	
IFNγ expression *in vitro*	+				+	+	+	+		+			+	+

Figure.3.4. Comparison between *in vivo* and *in vitro* IFNγ mRNA expression in response to the peptide pools. Peptide pool-induced IFNγ mRNA expression gene was detected by real-time PCR in biopsy samples (open bars) and from *in vitro* stimulated PBMC (closed bars). The "+" and "x" indicate positive responses to the peptide pool *in vitro* and *in vivo*, separately.

3.4.4. Determination of *in vivo* cellular immune responses in four ponies at 6 months post- infection with EIAV

The gp90-specific cellular immune responses *in vivo* were determined at 6 month post challenge following the procedures described above. Three of the ponies H40 (peptide pools H and 8), H41 (peptide pools D,E,H,1,3,4,8) and H42 (peptide pools A,C,D,F,I,J,2,8,9) responded to gp90 peptide pools whereas H43 did not. Single peptide reactivities were predicted, as shown in grey boxes in Figure 3.5, and individual peptides were injected into the neck of the corresponding ponies. Positive physical reactions to these individual peptides (indicated with * in Figure 3.5) were confirmed by increased CD3 and IFNγ gene expression (data not shown). As shown in Figure 3.5, all the positive peptide pools contained at least one confirmed positive single peptide. Although some of the peptides from the EV0, EV6, and EV13 viruses share similar sequences (Craigo et al., 2007d), the ponies only recognized specific peptides from one virus. For example, H42 recognized single peptide #34 from EV6, but not single peptide #34 from EV0 or EV13 that share similar amino acid sequences, demonstrating the specificity of this *in vivo* assay (Table 1).

A

H40

	A	B	C	D	E	F	G	H	I	J	
1	*14*	*15*	*18*	*19*	*28*	*21*	*23*	*24*	*27*	*20*	0
2	*30*	*31*	*32*	*34*	*35*	*37*	*38*	3	4	14	0
3	15	18	19	20	21	23	22	27	24	38	0
4	29	30	31	32	33	34	35	36	37	28	0
5	39	40	41	1	3	5	7	9	11	13	0
6	19	17	15	21	23	25	27	29	31	33	0
7	35	37	39	41	43	2	4	6	8	10	0
8	12	14	16	18	20	22	24	26 *	28	30	125
9	32	34	36	38	40	42	44				0
	0	0	0	0	0	0	0	100	0	0	

Areas of palpable infiltrations(mm2)

Areas of palpable infiltrations(mm2)

B

H41

	A	B	C	D	E	F	G	H	I	J	
1	*14*	*15*	*18*	*19*	*28 **	*21*	*23*	*24*	*27*	*20*	100
2	*30*	*31*	*32*	*34*	*35*	*37*	*38*	3	4	14	0
3	15	18	19	20 *	21	23	22	27	24	38	100
4	29	30	31	32	33 *	34	35	36 *	37	28	150
5	39	40	41	1	3	5	7	9	11	13	0
6	19	17	15	21	23	25	27	29	31	33	0
7	35	37	39	41	43	2	4	6	8	10	0
8	12	14	16	18 *	20 *	22	24	26 *	28	30	250
9	32	34	36	38	40	42	44				0
	0	0	0	100	100	0	0	200	0	0	

Areas of palpable infiltrations(mm2)

Areas of palpable infiltrations(mm2)

C

H42

	A	B	C	D	E	F	G	H	I	J	
1	*14*	*15*	*18*	*19*	*28*	*21*	*23*	*24*	*27*	*20*	0
2	*30*	*31*	*32*	*34 **	*35*	*37*	*38*	3	4 *	14	50
3	15	18	19	20	21	23	22	27	24	38	0
4	29	30	31	32	33	34	35	36	37	28	0
5	39	40	41	1	3	5	7	9	11	13	0
6	19	17	15	21	23	25	27	29	31	33	0
7	35	37	39	41	43	2	4	6	8	10	0
8	12 *	14	16	18 *	20	22	24	26	28 *	30 *	200
9	32 *	34	36 *	38 *	40	42 *	44				50
	100	0	100	100	0	100	0	0	100	100	

Areas of palpable infiltrations(mm2)

Areas of palpable infiltrations(mm2)

Figure.3.5 Cellular epitope recognition in newly infected ponies. The original peptide pools (Figure 3.1A) were supplemented with those peptides unique to the EV6 and EV13 viruses. Those peptides in *italics* are unique to EV6 and those underlined are unique to EV13. Palpable infiltration (mm^2) were shown on the bottom and left sides of each figure. Intersecting pools (gray shading) identify possible peptide specificities. Single peptides with * are the peptides which could elicit positive responses *in vivo*.

Table 3.1 Peptides sequence comparisons among EV0, EV6 and EV13.

	#34 peptide sequence[1]	CD3 gene expression[2]	IFNγ gene expression[2]
EV0	PIFYTCNFTNITSCNNEPII	0	0
EV6	PIFYTCNFTNITSCNNESII	4	16
EV13	PMFYTCNFTSITSCNDESIT	0	0

[1]Underscores indicate amino acid differences from the original EV0 peptide.
[2] Relative gene expression determined using RT-PCR

3.5 Discussion

For over a hundred years, the delayed-type hypersensitivity reaction has been used for the diagnosis of both human and bovine tuberculosis (TB). After the intradermal injection of a small amount of antigens in sensitized individuals, swelling and induration become apparent between 24 and 72 hours. However, it was not until the 1940s when Landsteiner and Chase were able to clarify that the reaction was mediated by cellular instead of the humoral immune responses (Black, 1999). More recently, specific protein epitopes have been identified which further refine this method (Whelan et al., 2010). The DTH response has been used to assess either CD4+ or CD8+ T cells response to a variety of antigens (Dietert et al., 2010; Poulter et al., 1982; Waksman, 1979). However, this approach has rarely been used in horses (Hall et al., 2004). Further, only limited efforts have been made to test the specificity and sensitivity of DTH responses to small peptides in large animals. By contrast, DTH testing has been widely used in mice (Ashbridge et al., 1992; Nishino et al., 1994) and guinea pigs (Estrada et al., 1992; Mackall et al., 1993), and humans (Kran et al., 2012; Sitz et al., 1997b; Sitz et al., 1999) to evaluate epitope-specific responses using small peptides. Here, we show that small peptides from EIAV gp90 can elicit DTH responses within 48hrs post injection in EIAV infected horses. In order to further characterize this DTH response in horses, we collected skin biopsies at the injection sites for histologogy and gene expression analysis. The histological examination showed an influx of $CD3^+$, $CD25^+$ T cells (Figure 3.3) within 48 hrs post-injection. Furthermore, we found this cellular influx was directly correlated with increased CD3 and IFNγ gene expression, a phenomenon not observed at injection sites receiving the saline control or peptides that failed to stimulate palpable reactions. By employing a peptide matrix

approach, followed by injection of individual peptides, it is possible to characterize cell mediated immune responses at the level of individual protein epitopes. This provides for the opportunity for comprehensive investigations of *in vivo* antigen-specific cellular immune responses in EIAV infected horses.

While DTH responsiveness is often associated with IFNγ production *in vitro* (Kapsenberg et al., 1991; Weynants et al., 1995), two peptide pools which induced DTH responses *in vivo* did not up-regulate IFNγ gene expressions *in vitro*. This disparity between *in vivo* and *in vitro* responses has also been reported in the case of HIV (Kvale et al., 2005). There are several possibilities for these differences. By virtue of the sampling method, PBMC represents a minor fraction of the total lymphocyte population. A low frequency of antigen-specific cells thus reduces the chance of including such cells in a blood sample. Since precursor expansion is required for the *in vitro* detection of specific immune responses, both an infrequent precursor population and the likelihood that the T cells will undergo apoptosis *in vitro* (Herbein et al., 1998; Shearer, 1998) further reduces the sensitivity of this assay. By contrast, the DTH response involves the active recruitment of antigen-specific T cells to the injection site, thus increasing the likelihood of a measureable response even with a low frequency population. We also observed the situation where peptide pools were recognized *in vitro*, but did not elicit a DTH response *in vivo*. Such discordant responses may reflect a situation where the effector cell produces IFNγ but not the other cytokines or chemokines required for a DTH response (Vestweber, 2007). Another possibility is that over time, the kinetics of development or loss of DTH compared to IFNγ secretion differ (Black et al., 2001). The significance of this dichotomy

between *in vitro* and *in vivo* responses in terms of protective immunity remains to be determined.

Three of the newly infected ponies used in this study exhibited variable clinical signs of EIA, characterized by fever, decrease in platelets, and increased virus load in plasma. There appears to be a relationship between virus replication and the number of the peptides recognized by these ponies. Thus, H42, which recognized multiple peptides, also had multiple febrile episodes. These febrile episodes are typically associated with a recurring high levels of viremia (Hammond et al., 1997). This indicates that the broader peptide recognition was associated with repeated exposure to the virus, thus likely expanding the pool of antigen-specific T cells. Since protective immunity eventually occurs in EIAV-infected horses, this approach provides the opportunity to identify the evolution of the protective mechanisms responsible for virus control in the EIAV infected ponies, and perhaps as well for other lentivirus (Addo et al., 2003). By contrast, H43, which did not show any clinical signs of EIA, did not recognize any peptides. We occasionally encounter this situation where a particular equid effectively controls EIAV replication while invariably having very weak antibody responses. Our current results indicate there is a similarly weak CMI reactions in these seemingly resistant animals. While the mechanism of this innate resistance remains unknown, it likely involves non-immunological mechanisms based on these results.

In summary, we described a method to assess EIAV envelope specific cellular immunity *in vivo*. Histological and gene expression analysis were used to confirm the cellular nature of this response. As such, this approach offers an alternative to *in vitro* detection of antigen-specific cellular immune responses.

3.6 Acknowledgements

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CHAPTER 4

Epitope shifting of gp90-specific cellular immune responses in EIAV infected ponies

4.1 Summary

EIAV has been used as a model to investigate protective mechanisms against lentiviruses. Unlike other lentiviruses, EIAV replication can be eventually controlled in most infected horses, leading to an inapparent carrier state free of overt clinical signs which can last for many years. Maintenance of this carrier state is absolutely dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce the recurrence of disease. However, the immune mechanisms that are responsible for this control of infection are not yet identified. As the resolution of the initial infection is correlated with the appearance of the virus specific CTL, it appears that cellular immune responses play an important role. Since the virus undergoes rapid mutation following infection, the adaptive immune response must also evolve to meet this challenge. In order to fully understand the evolution of cellular immune responses, we synthesized forty-four peptides, spanning the entire surface unit protein (gp90) of EIAV, and monitored the peptide-specific T cells responses *in vivo* over six months following infection. Peptides were injected intradermally and punch biopsies were collected for real-time PCR analysis to monitor the cellular immune responses *in vivo*. Similar to the CMI response to HIV infection, peptide-specific T cell recognition patterns in EIAV newly infected ponies changed over time. While some peptides were recognized throughout the sampling period, other peptides were only recognized at the later time points. Also, the response to some

specific peptides disappeared after 6 months post infection. By contrast, peptide recognition by an inapparent carrier (D64) was more stable.

4.2 Introduction

Equine infectious anemia virus (EIAV) has been used as a model for investigating the underlying protective immune mechanisms against lentiviruses (Craigo and Montelaro, 2011). Following infection, most horses control their initial EIAV infection leading to an inapparent carrier state (Hammond et al., 2000; Harrold et al., 2000). Immune responses play an important role in controlling EIAV infection (Mealey et al., 2001b; Perryman et al., 1988) since the administration of immunosuppressive drugs to inapparent carriers can induce the recurrence of disease (Craigo et al., 2002a). The development of protective immunity in EIAV-infected horses is protracted and often requires 6 – 12 months to become established (Hammond et al., 2000; Harrold et al., 2000). Likewise, an attenuated vaccine strain $EIAV_{D9}$ provided protection against homologous virus challenges (Craigo et al., 2007b), but optimum protective immunity was not seen until six months post vaccination. Analyses of longitudinal serum samples from EIAV-infected horses displayed an evolution of envelope-specific antibodies responses as measured by changes in avidity, conformational recognition, and neutralization titers in EIAV infected horses (Hammond et al., 1997; Hammond et al., 1999).

Cellular immune responses also appear important in controlling EIAV infections. The appearance of EIAV-specific cytotoxic T lymphocytes (CTL) correlates with the control of the initial viremia (McGuire et al., 2000). Additionally, CTL responses are detected in inapparent carriers during the chronic phase of EIAV infection (Chung et al.,

2005; Mealey et al., 2003a; Zhang et al., 1999). Both T helper (Th) and CTL epitopes have been identified in the EIAV envelope protein of EIAV-infected horses (Tagmyer et al., 2008a). Various studies have demonstrated EIAV Gag and Pol specific CTL responses (Lonning et al., 1999; Mealey et al., 2005b) and identified broadly reactive T-cell epitopes (Chung et al., 2004, 2005; McGuire et al., 2000). Nevertheless, the identification of epitope-specific cellular immune responses that correlate with protection has proven elusive (Mealey et al., 2009). Further, not all potential epitopes in an antigenically complex viral antigen can be recognized by CD4 or CD8 T cells (Gallimore et al., 1998b). Also, not all recognized epitopes elicit equal responses. Typically, T cells responses focus on one or a few epitopes (immunodominant epitopes) and the remainder of the epitopes (subdominant epitopes) elicit limited responses (Yewdell and Bennink, 1999b). Most acute viral infections involve the early recognition of immunodominant epitopes leading to the eradication of the infection (Chisari and Ferrari, 1995; Perelson et al., 1993). By contrast, in chronic viral infections there is a shift in T cell recognition from immunodominant to subdominant epitopes (Fuller et al., 2004; Turner et al., 2005; Wherry et al., 2003). This epitope shift is evidenced by increases in the specificity, breadth and magnitude of T cell responses over time (Moskophidis et al., 1993).

Here, we monitored the peptide specificity of the cellular immune responses during early EIAV infection. Over the initial six month post-infection, EIAV envelope specific T cells responses were detected *in vivo*. Our results indicated that EIAV envelope specific T cells undergo a shift in epitope recognition over time. This shift in epitope recognition may be associated with the control of viral replication and the establishment of the inapparent carrier state.

4.3 Materials and Methods

4.3.1 Ponies, virus and experimental challenge

Sixteen ponies of mixed age and gender were used in this study. MHC haplotypes were determined using polymorphic microsatellites, as previously described (Tseng, Miller et al. 2010). Five ponies (G35, L132, H46, H38 and H32) shared the same MHC microsatellite allele 219-252-280-172-247, three of the ponies (L126, L132 and I28) shared the allele 211-260-268-166-247, and another three ponies (D49, H36 and H32) shared the same MHC microsatellite allele 211-260-278-174-241 (Table 4.1). An inapparent carrier infected with EIAV for more than six years was also included for comparisons.

The $EIAV_{D9}$ was used as the challenge virus. The $EIAV_{D9}$ stain contains two stop codons and nine base pair deletion into the S2 gene of the infectious molecular clone $EIAV_{UK}$ (Craigo et al., 2007b). Previous work has shown that that ponies infected with $EIAV_{D9}$ develop protective immunity against EIAV strains with a homologous gp90 sequence (Craigo et al., 2007b).

Ponies were inoculated intravenously with10^3 $TCID_{50}$ of $EIAV_{D9}$ (Craigo, Durkin et al. 2007). All ponies were monitored daily for clinical symptoms of EIA throughout the course of the 6 months study. Plasma samples from all animals were collected weekly and stored at − 80ºC for later use to determine the levels of plasma viral RNA, as previously described (Craigo, Durkin et al. 2007).

All animals were handled under the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture, according to protocols approved by the University of Kentucky Institutional Animal Care and Use Committee.

Table 4.1 MHC haplotypes

Horse ID	Virus	UMN-JH34-2-COR112-COR113-UM011-COR114	UMN-JH34-2-COR112-COR113-UM011-COR114
G35	$EIAV_{D9}$	219-252-280-172-247	207-254-268-174-235
L126	$EIAV_{D9}$	211-260-268-166-247	211-260-268-166-247
L132	$EIAV_{D9}$	219-252-280-172-247	211-260-268-166-247
F31	$EIAV_{D9}$	205-252-274-168-243	203-260-266-168-249
I28	$EIAV_{D9}$	215-252-260-168-243	211-260-268-166-247
H46	$EIAV_{D9}$	221-268-278-174-241	219-252-280-172-247
D49	$EIAV_{D9}$	211-260-278-174-241	211-258-274-164-237
H38	$EIAV_{D9}$	219-252-280-172-247	223-252-268-166-247
H36	$EIAV_{D9}$	211-260-278-174-241	205-252-274-168-243
H32	$EIAV_{D9}$	211-260-278-174-241	219-252-280-172-247
D55	$EIAV_{D9}$	215-252-260-168-243	211-258-274-164-237
I35	$EIAV_{D9}$	205-262-270-184-245	221-262-270-172-237
J30	$EIAV_{D9}$	197-248-270-184-245	219-244-268-172-249
L124	$EIAV_{D9}$	221-262-270-172-237	221-238-264-180-243
I33	$EIAV_{D9}$	219-236-266-168-249	203-260-266-178-241
L128	$EIAV_{D9}$	219-250-266-170-245	211-262-270-184-245

4.3.2 Production of synthetic peptides and construction of peptide pools matrix

Forty-four overlapping peptides of 20 amino acids in length spanning the entire gp90 of $EIAV_{D9}$ strain were synthesized (Table 4.2) and used to construct peptide pools (Figure4.1). All peptides were HPLC-purified and dissolved in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) at a stock concentration of 2 mg/ml. (Tagmyer et al., 2007). The optimal working concentration for individual peptides was determined by serially diluting a known positive peptide as previously described (Liu et al., 2012). A dose of 0.5µg in 100ul saline was used for individual peptides and when incorporated into the peptide pools.

	A	B	C	D	E	F	G
1	1	3	5	7	9	11	13
2	15	17	19	21	23	25	27
3	29	31	33	35	37	39	41
4	43	2	4	6	8	10	12
5	14	16	18	20	22	24	26
6	28	30	32	34	36	38	40
7	42	44					

Figure 4.1 Matrix of EV0 peptides. Forty-four peptides from $EIAV_{D9}$ were synthesized. In total, 14 peptides pools were constructed and each single peptide could be found in two different peptide pools.

Table 4.2 Gp90 peptide Sequences

EV0 peptide No.	EV0 peptide sequence	Region[1]
1	MVSIAFYGGIPGGISTPITQ	I
2	PGGISTPITQQSEKSKCEEN	I
3	QSEKSKCEENTMFQPYCYNN	I
4	TMFQPYCYNNDSKNSMAESK	I
5	DSKNSMAESKEARDQEMNLK	I
6	EARDQEMNLKEESKEEKRRN	I
7	EESKEEKRRNDWWKIGMFLL	I
8	DWWKIGMFLLCLAGTTGGIL	I
9	CLAGTTGGILWWYEGLPQQH	I
10	WWYEGLPQQHYIGLVAIGGR	I
11	YIGLVAIGGRLNGSGQSNAI	I
12	LNGSGQSNAIECWGSFPGCR	I
13	ECWGSFPGCRPFQNYFSYET	I
14	PFQNYFSYETNRSMHMDNNT	I
15	NRSMHMDNNTATLLEAYHRE	II
16	ATLLEAYHREITFIYKSSCT	II
17	ITFIYKSSCTDSDHCQEYQC	II
18	DSDHCQEYQCKKVNLNSSDS	II
19	KKVNLNSSDSSNPVRVEDVM	II
20	SNPVRVEDVMNTTEYWGFKW	II
21	NTTEYWGFKWLECNQTENFK	II
22	LECNQTENFKTILVPENEMV	II
23	TILVPENEMVNINDTDTWIP	II
24	NINDTDTWIPKGCNETWARV	II
25	KGCNETWARVKRCPIDILYG	II
26	KRCPIDILYGIHPIRLCVQP	II
27	IHPIRLCVQPPFFLVQEKGI	II
28	PFFLVQEKGIANTSRIGNCG	II
29	ANTSRIGNCGPTIFLGVLED	III
30	PTIFLGVLEDNKGVVRGNYT	III
31	NKGVVRGNYTACNVSRLKIN	III
32	ACNVSRLKINRKDYTGIYQV	III
33	RKDYTGIYQVPIFYTCNFTN	III
34	PIFYTCNFTNITSCNNEPII	III
35	ITSCNNEPIISVIMYETNQV	III
36	SVIMYETNQVQYLLCNNNNS	III
37	QYLLCNNNNSNNYNCVVQSF	III

Table 4.2 (continued)

38	NNYNCVVQSFGVIGQAHLEL	III
39	GVIGQAHLELPRPNKRIRNQ	III
40	PRPNKRIRNQSFNQYNCSIN	III
41	SFNQYNCSINNKTELETWKL	III
42	NKTELETWKLVKTSGITPLP	III
43	VKTSGITPLPISSEANTGLI	III
44	ISSEANTGLIRHKRDFGISA	III

Footnote: 1: Gp90 are segmented into I,II,III regions equally

4.3.3 Determination of gp90 specific cellular immune responses over time *in vivo*

Identification of gp90-specific cellular immune responses *in vivo* was performed, as described (Liu et al., 2012). Peptide pools or single peptides were injected intradermally into the neck of each pony. After 48hrs, the palpable infiltrations were measured at each injection site and a 2mm skin biopsy was collected and stored in RNALater® (Ambion, Austin, TX). Skin biopsies were later homogenized using a Retsch MM301 Ball Mill (Newtown, PA) and the suspended cell-lysates transferred to a 1.5ml eppendorf tube containing RNA-STAT 60 (Tel-Test). Total RNA was isolated and 1 µg of RNA was dissolved in 41.5µl nuclease-free water (Qiagen, Valencia, CA) and reverse transcribed into cDNA. The cDNA was stored in -20^{o}C until later use for real-time PCR or spectratyping.

Intron-spanning TaqMan primers and probes for equine IFNγ were used for real-time PCR, as previously described (Liu et al., 2011b). PCR amplification efficiencies were determined using Linreg (Ramakers et al., 2003) and only samples with amplification efficiencies above 99% were included for further analyses. Beta-glucuronidase (β-GUS) was used as the housekeeping gene. The saline injection site for each horse used as the

calibrator (Liu et al., 2011b) and the relative quantity (RQ) for each target gene was calculated as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001b). RQ values greater than 2 were considered positive.

4.3.4 Determine gp90 specific T cells repertoire over time *in vivo*

The spectratyping method was used to identify peptide-specific clonal T cell expansion in the injection sites . This assay was performed at the University of California at Davis in the laboratory of Dr. Johanna Watson. Briefly, an equine T cell receptor (TCR) β chain constant region-specific primer and twenty-four equine TCR Vβ-specific primers corresponding to the complementarity-determining region 3 (CDR3) of the equine TCR β chain were designed. Total RNA isolated from the intradermal injection sites was converted into cDNA and used for the spectratyping. The PCR reactions were incubated in triplicate wells at 95ºC for 15 min, followed by 35 cycles of 94ºC for 30s, 54ºC for 30s and 72ºC for 30s. An ABI 3730 sequencer was used for determining amplicon length and the size distribution analyzed using STRAND (Toonen and Hughes, 2001).

4.3.5 Stimulation and measurement of effector cells *in vitro*

Venous blood was collected via aseptic venipuncture into heparinized tubes, and peripheral blood mononuclear cells (PBMC) isolated using Ficol-Paque PlusTM (Amersham Biosciences,Piscataway, NJ) gradient centrifugation. After washed with PBS for three times, PBMC were cryopreserved and stored in liquid nitrogen for later use.

Cryopreserved PBMC were thawed and approximately 2 x 10^6 were pulsed with 5ug/ml of the peptides in 1ml fresh RPMI-1640 (Gibco, Grand Island, NY) supplemented

with 2.5% (v/v) fetal equine serum (FES, Sigma, St. Louis, MO), 2mM glutamine (Sigma), 100U/ml penicillin/streptomycin (Sigma), and 55 µM 2-mercaptoethanol (GIBCO, Grand Island, NY). The peptide-stimulated PBMC were incubated *in vitro* for 4 days in a 24 well plate at 37 ℃ in a 5% CO2 incubator. The cells were then pelleted and re-suspended in 1ml of RNA-STAT 60 (Tel-Test) for further analysis of gene expressions by real-time PCR, as described above.

4.4 Results

4.4.1 Clinical Response to infection

Each pony seroconverted by 35 days post infection, as determined by commercial ELISA kits (data not shown). There were no changes in rectal temperature (Figure 4.2A) or platelet counts (Figure 4.2B) in any of the ponies post-infection. A low level of $EIAV_{D9}$ replication, averaging 10^3–10^4 RNA copies/ml plasma, was detected during the first six months post infection (Figure 4.2B).

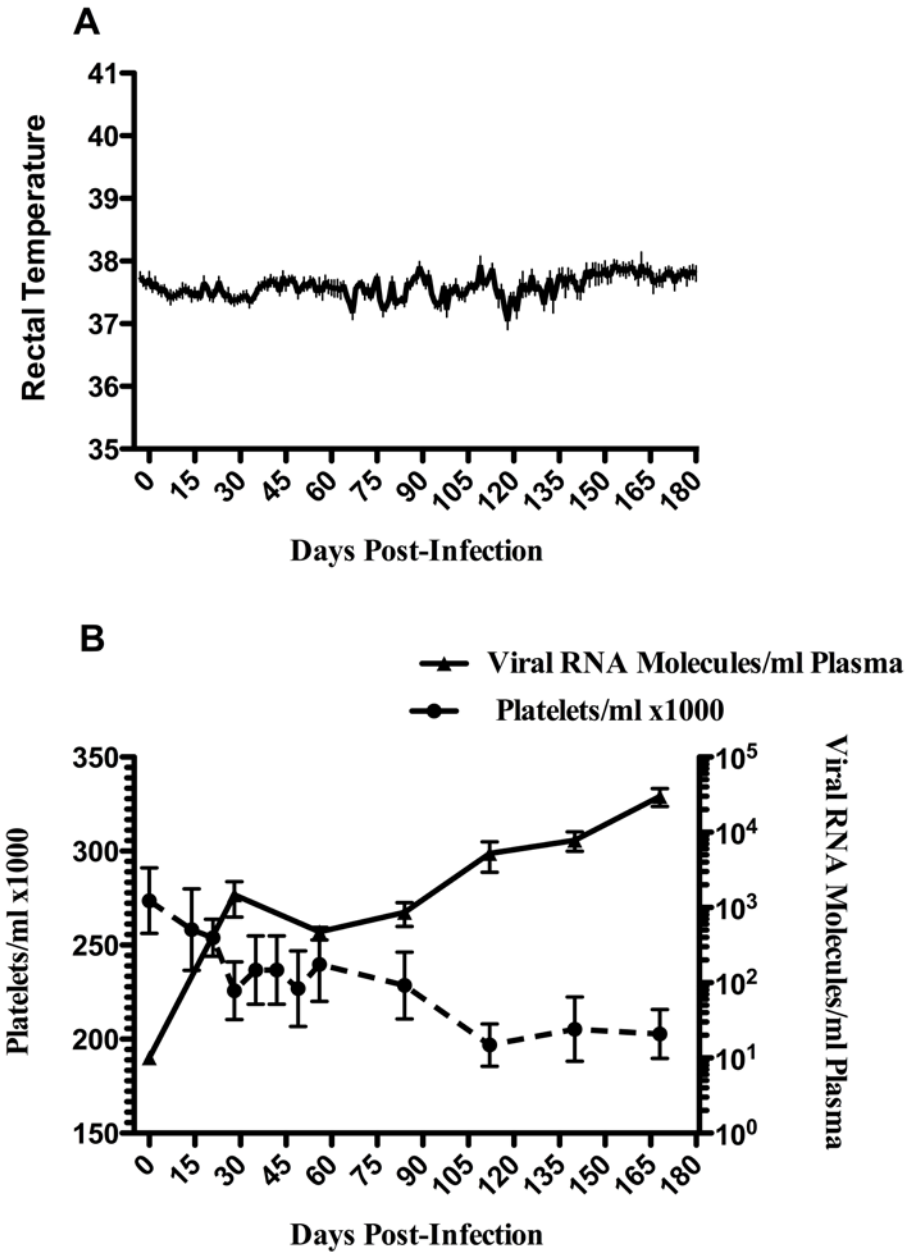


Figure 4.2 Clinical and virological profiles of $EIAV_{D9}$ infected ponies. A) Rectal temperature (°C) was measured daily in all $EIAV_{D9}$ infected ponies. The average rectal temperature was calculated and plotted. B) Viral p26 copies in the plasma (right axis) and platelet counts (left axis) were determined at 3 week interval. The average p26 copies and platelet counts for the 16 experimentally infected ponies are shown.

4.4.2 Determination of gp90-specific cellular immune responses in $EIAV_{D9}$ infected ponies *in vivo*

Of the 16 ponies tested, 11 ponies showed gp90-specific responses following intradermal inoculations. Screening all ponies with 44 EV0 peptides indicated that not all peptides were recognized equally (Figure 4.3). Peptides located in regions I (#2, #6, #8, #11, #14), II (#16, #21, #22, #23, #26, #28) and III (#30, #33, #34, #35, #36, #38, #41, #42, #44) could elicit positive responses. Peptides #36 and #38 which are located in region III were the most recognized peptides, by 4 and 5 ponies, respectively.

EIAV gp90-specific IFNγ gene expression in the intradermal sites was determined at 1, 3 and 6 months post infection (Figure 4.4). Our results indicated that gp90-specific T cells responses underwent both expansion and contraction. One month after infection, 7 ponies exhibited a gp90-specific IFNγ response. Five of the ponies recognized only one peptide, and two ponies recognized two peptides (Figure 4.4A). Peptides #36 and #38 were recognized by 2 and 4 ponies, respectively. Three months after infection, there were more peptides in the middle and amino-terminus of gp90 recognized (Figure 4.4B). Six months post infection, most ponies recognized more than one peptide and the recognized peptides spanned the entire sequence of gp90 (Figure 4.4C). While some peptides were recognized throughout the sampling period (L132 #36), other peptides were only recognized at the later time points (G35 #26 #33 #36). Also, the response to some specific peptides disappeared by 6 months post infection (D49 #38).

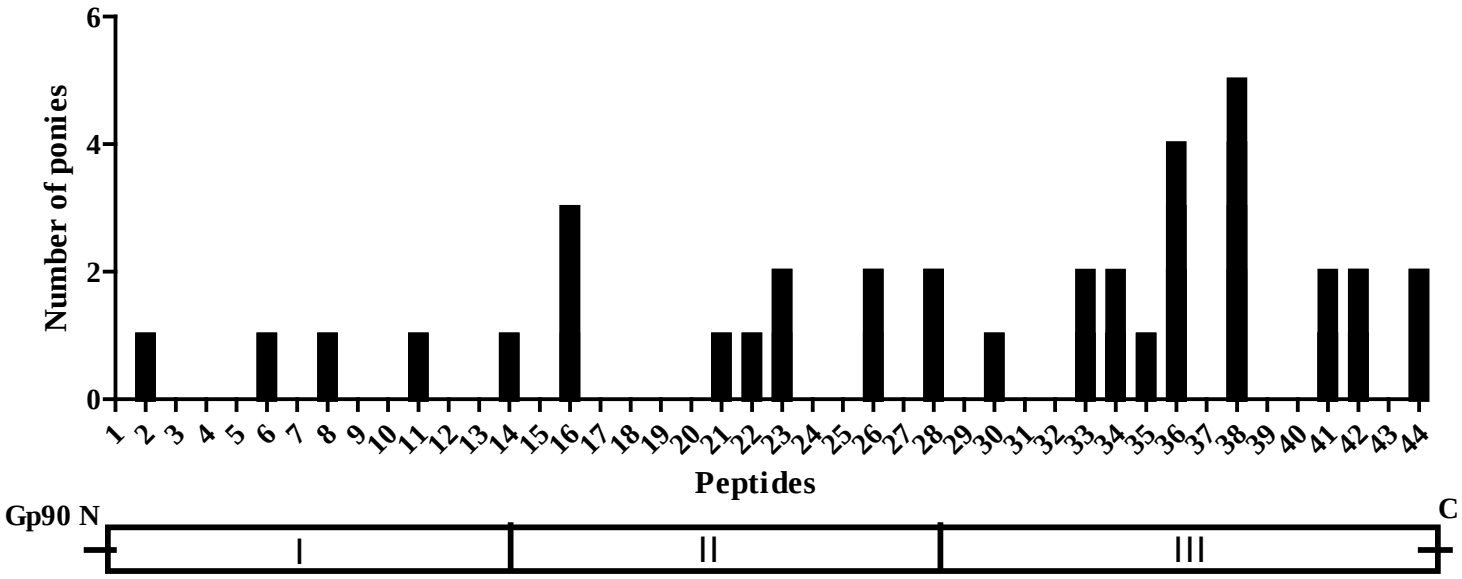


Figure 4.3 Summary of peptides recognition pattern in $EIAV_{D9}$ infected ponies. Eleven of the 16 ponies showed gp90 peptide-specific responses during the six month period of observation. The figure shows the accumulated recognition pattern for each of the peptides tested.

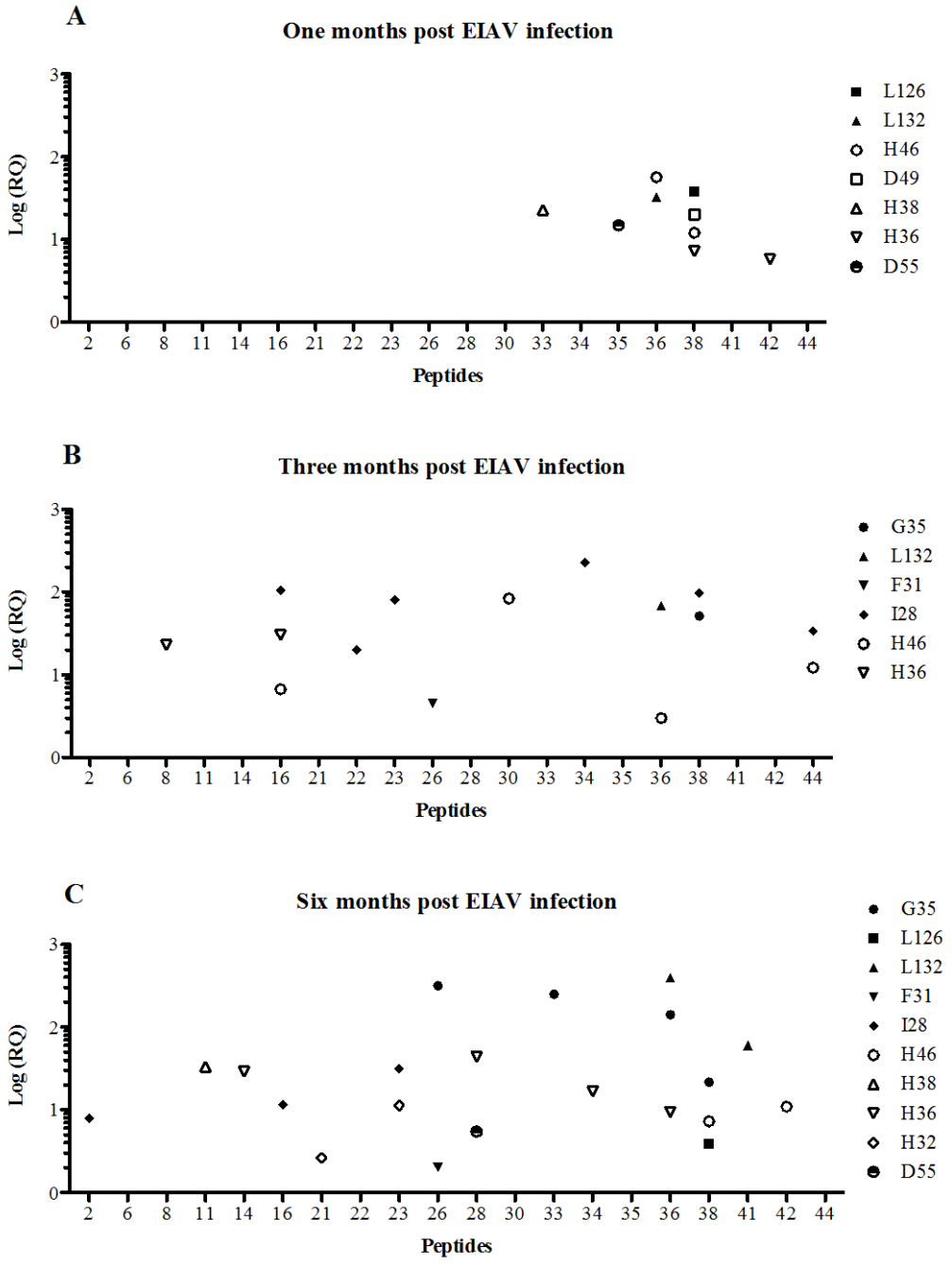


Figure 4.4 IFNγ gene expressions in peptides injection sites in $EIAV_{D9}$ infected ponies. Ponies were tested at 1 (A), 3 (B) and at 6 (C) months post-infection. IFNγ gene expression was determined using relative quantitation (RQ) and the results were log transformed. Each point represents an individual pony's response to the peptide.

In comparison, an inapparent carrier which has been infected with EIAV for more than six years showed more stable recognition. Thus, peptide C, E, F, G, 3, 4, and 6 elicited a similar *in vivo* response over a 3 year period (Figure 4.5A). Furthermore, injection of predicted single peptides (Grey boxes) demonstrated that this inapparent carrier recognized the same peptides (4, 12, 37, 38 and 40) and produced IFNγ mRNA at the injection site at both sampling times (Figure 4.5B).

4.4.3 T cells are recruited to the injection sites.

Further investigation of the diversity of recruited T cells was evaluated by measuring the heterogeneity of the TCR CDR3 region at the injection site using spectratyping, The total T cell CDR3 repertoire will typically follow a Gaussian distribution whereas the recruitment/expansion of specific T cells clones to the injection site will be represented as polyclonal skewed or oligoclonal distribution (Figure 4.6A). CDR3 diversity was determined at the positive reaction sites following peptide #38 injection in pony H46 (Figure 4.6B). Following injection of the peptide, T cells with 22 of the 24 TCR Vβ families were recruited to the site. Since not all Vβ families are represented, and within specific Vβ families there is both clonal and oligoclonal responses, these results are consistent with this being a T cell-specific response. By contrast, only three TCR Vβ families were present in the saline only injection site, likely representing resident cells. The breadth of T cells recruited to the injection sites was correlated with the magnitude of peptide specific IFNγ response (Figure 4.6C). Also, there was evidence of an overall reduction both in Vβ family utilization and the expression of IFNγ mRNA as the infection progressed.

A

	A	B	C	D	E	F	G	Year 2010	Year 2013
1	1	3	5	7	9	11	13	0	0
2	15	17	19	21	23	25	27	0	0
3	29	31	33	35	37	39	41	49	100
4	43	2	4	6	8	10	12	50	100
5	14	16	18	20	22	24	26	0	0
6	28	30	32	34	36	38	40	625	500
7	42	44						0	0
Year 2010	0	0	150	0	100	150	300		
Year 2013	0	0	100	0	150	400	225		

Areas of palpable infiltrations(mm^2)

Areas of palpable infiltrations(mm^2)

B

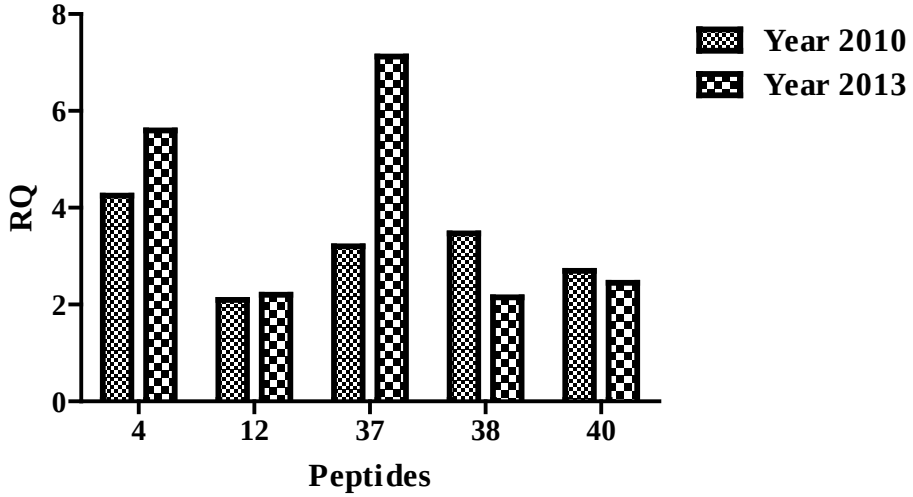
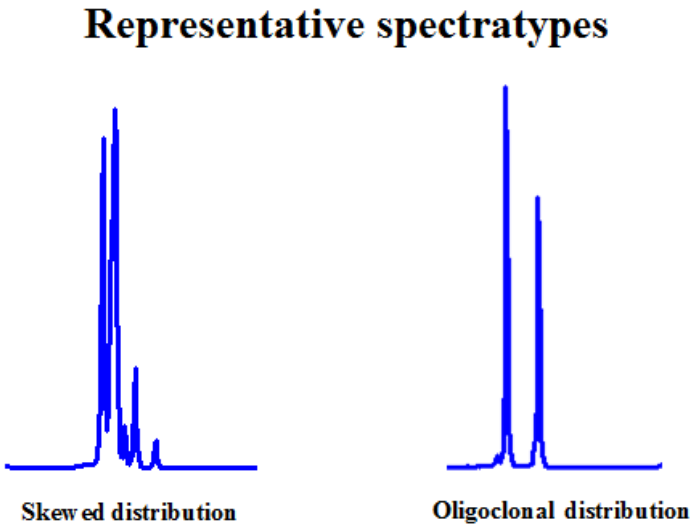


Figure 4.5 Stable peptide recognition is seen in an inapparent carrier. A) Peptide pools were screened in an inapparent carrier in 2010 and 2013. B) Those peptides eliciting a positive response were biopsied and IFNγ gene expression determined using RT-PCR.

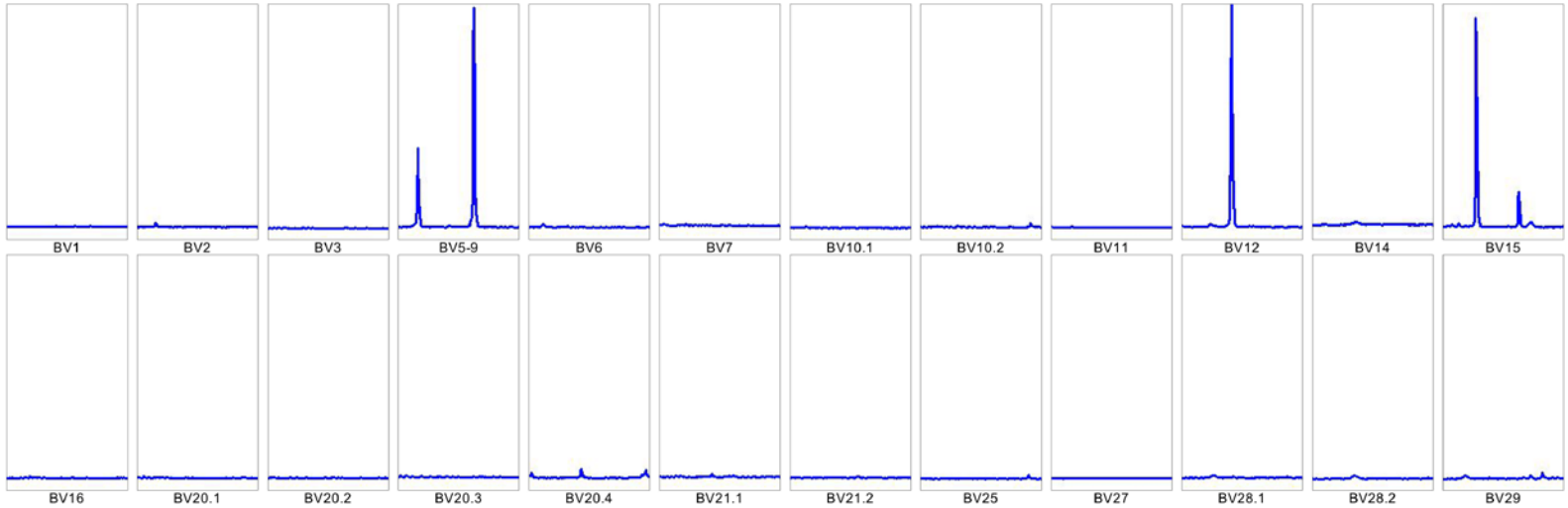
Figure 4.6. Spectratyping analysis of intradermal injection sites indicates the recruitment of peptide-specific T cells. A) When T cell clones undergo clonal expansion, skewed distribution or oligoclonal distribution will be detected. B) Peptide specific T cells repertoires in one pony which showed positive immune responses against peptide #38 determined by skin testing assays. Skin biopsies from both saline and #38 peptide injection sites were collected and processed for spectratyping. In total, 24 primer pairs spanning equine TCR b chain were used. C) Left axis shows IFNγ gene expression as determined by real time PCR of punch biopsies. Right axis shows the percentage of T cell clones recruited to the injection sites.

A

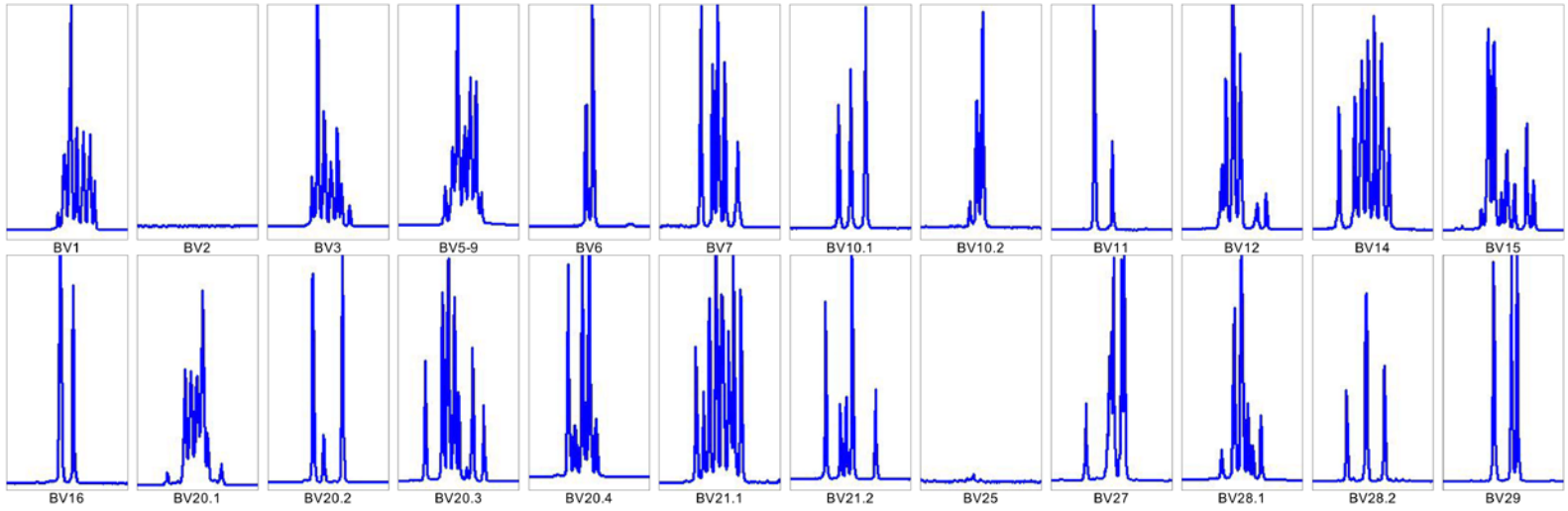


B

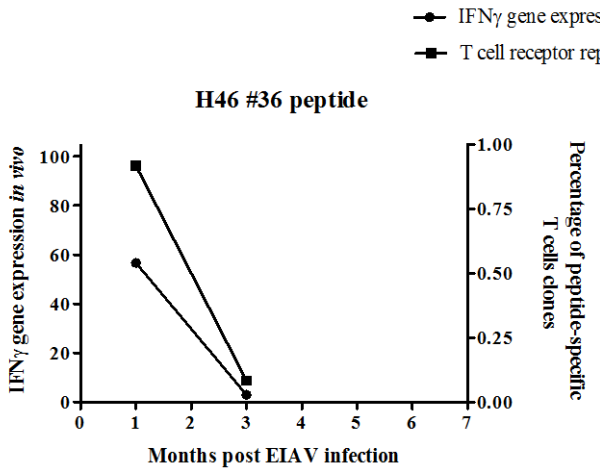
Saline

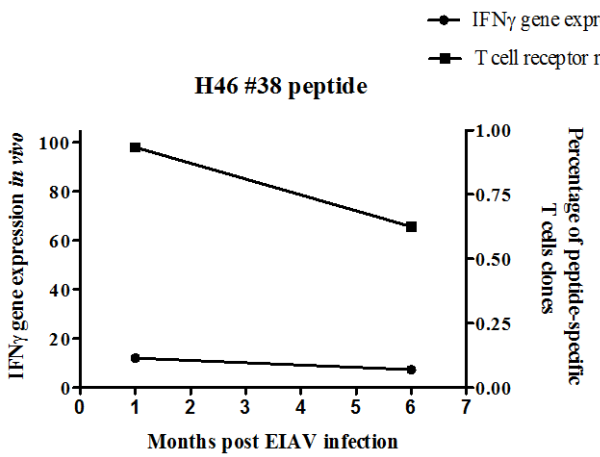

BV1
BV2
BV3
BV5-9
BV6
BV7
BV10.1
BV10.2
BV11
BV12
BV14
BV15
BV16
BV20.1
BV20.2
BV20.3
BV20.4
BV21.1
BV21.2
BV25
BV27
BV28.1
BV28.2
BV29

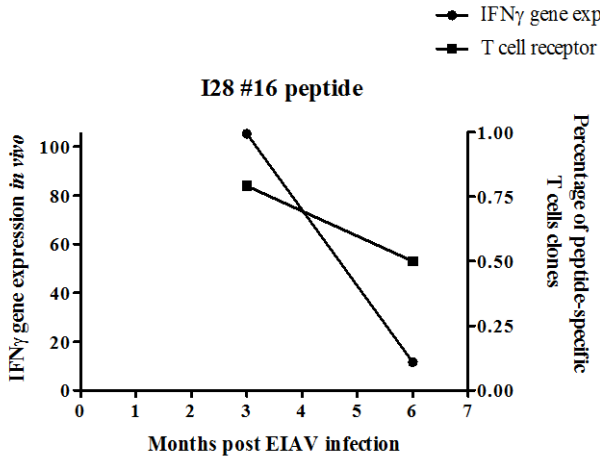
#38

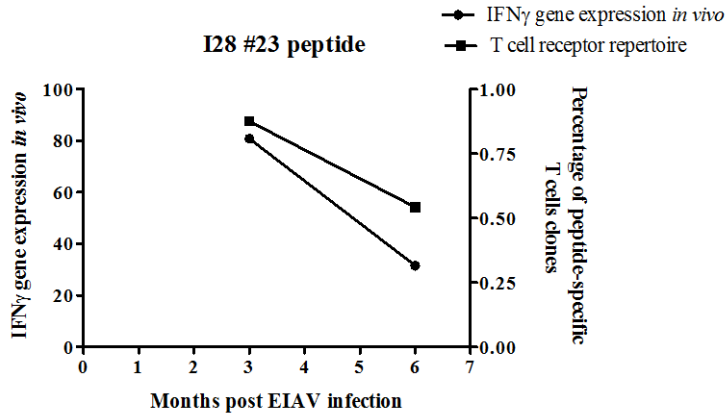

BV1
BV2
BV3
BV5-9
BV6
BV7
BV10.1
BV10.2
BV11
BV12
BV14
BV15
BV16
BV20.1
BV20.2
BV20.3
BV20.4
BV21.1
BV21.2
BV25
BV27
BV28.1
BV28.2
BV29

C


IFNγ gene expression in vivo
T cell receptor repertoire
H46 #36 peptide
IFNγ gene expression in vivo
Percentage of peptide-specific T cells clones
Months post EIAV infection

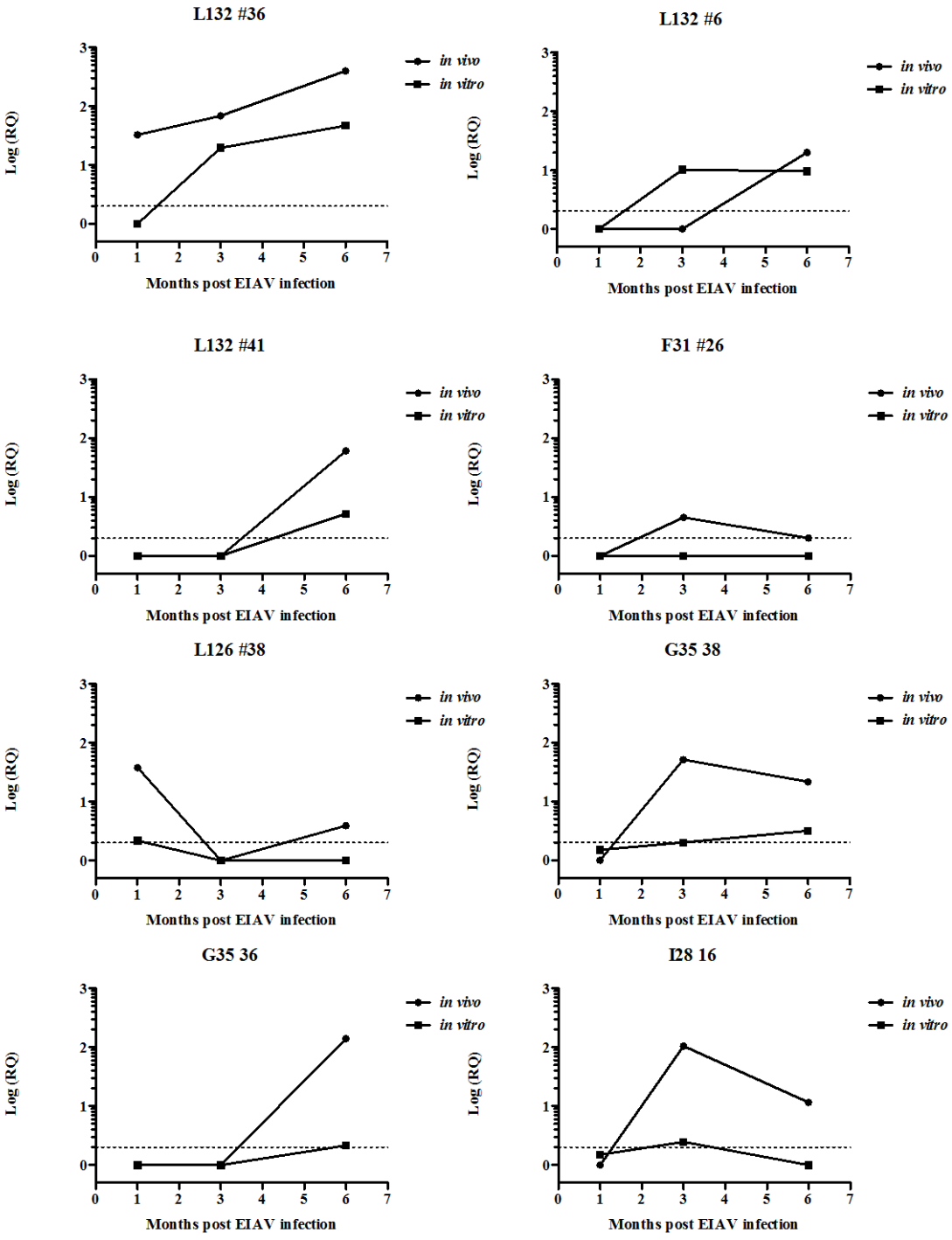

IFNγ gene expression in vivo
T cell receptor repertoire
H46 #38 peptide
IFNγ gene expression in vivo
Percentage of peptide-specific T cells clones
Months post EIAV infection


IFNγ gene expression in vivo
T cell receptor repertoire
I28 #16 peptide
IFNγ gene expression in vivo
Percentage of peptide-specific T cells clones
Months post EIAV infection


IFNγ gene expression in vivo
T cell receptor repertoire
I28 #23 peptide
IFNγ gene expression in vivo
Percentage of peptide-specific T cells clones
Months post EIAV infection

4.4.4 Gp90-specific cellular immune responses in $EIAV_{D9}$ infected ponies *in vitro*

Our results indicated that in newly infected ponies, gp90-specific T cells responses underwent expansion and contraction during the first six months post EIAV infection. In order to further confirm these changes, we also measured the peptide-specific T cell responses *in vitro* (Figure 4.7). A total of 13 peptides were selected and tested in 6 ponies at 1, 3 and 6 months post infection. In general, *in vivo* and *in vitro* assays showed similar peptide-specific T cells recognition patterns overtime. The *in vivo* assay detected peptide-specific responses sooner compared to the *in vitro* assay. For example, peptide #36 was recognized by L132 at 3 and 6 months post infection (both *in vivo* and *in vitro*), but only the *in vivo* response was detected at 1 month post infection.


L132 #36
L132 #6
L132 #41
F31 #26
L126 #38
G35 38
G35 36
I28 16
Log (RQ)
Months post EIAV infection
in vivo
in vitro

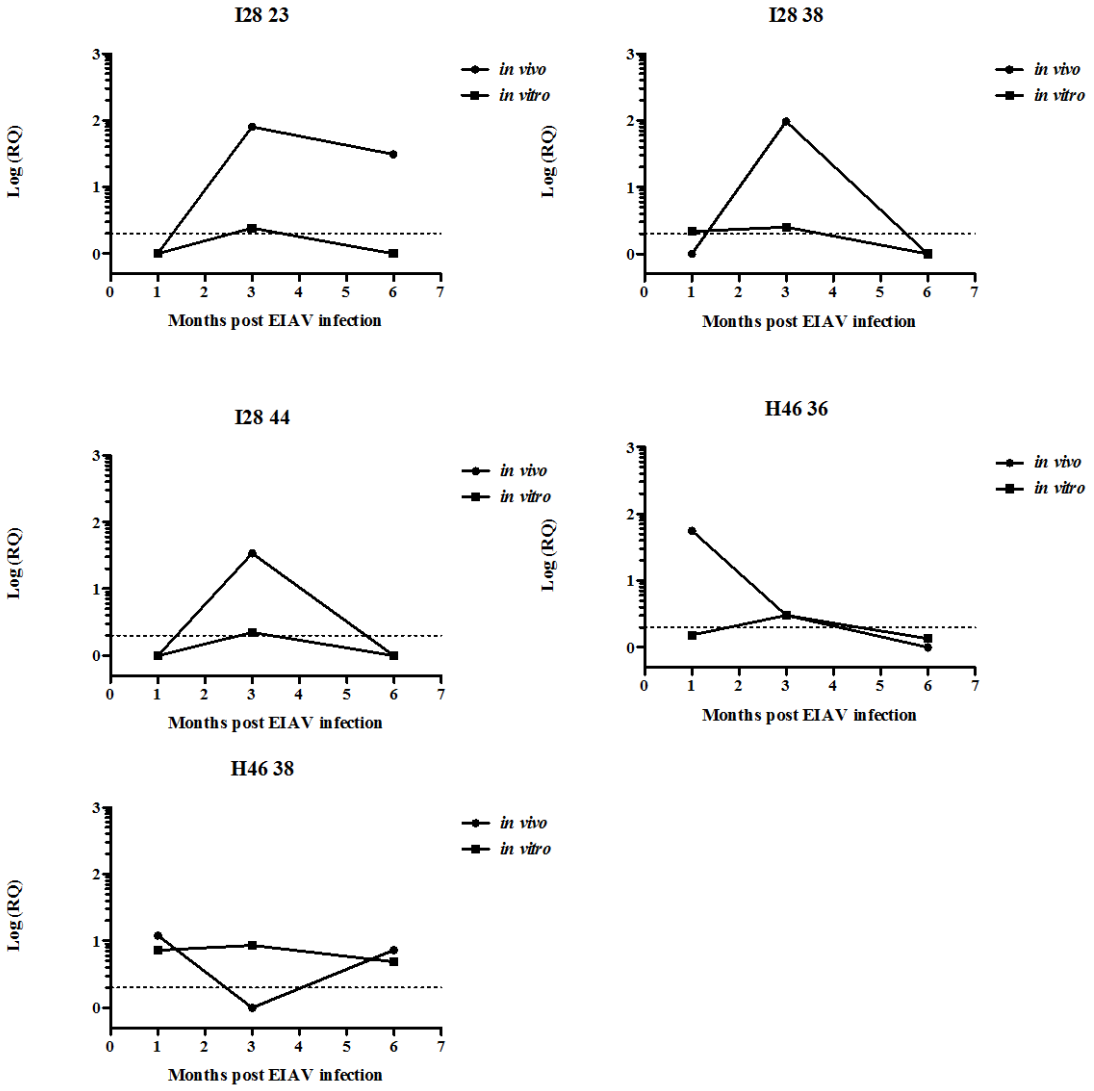


Figure 4.7 IFNγ gene expressions detected by *in vivo* or *in vitro* assays. PBMC were cultured with 5ug/ml specific peptides for 4 days for the determination of IFNγ gene expression. Relative expression (RQ) over 2 was viewed as positive and the dotted line indicates this threshold.

4.5 Discussion

The ability of lentiviruses to alter their antigenicity allows for their persistence in the infected host (Burns and Desrosiers, 1994; Craigo and Montelaro, 2010; Payne et al., 1987). To counter this antigenic variation, the immune responses must also adapt (Nowak et al., 1995a). Thus, in EIAV-infected horses, antigenic variation in the envelope gp90 results in escape variants that evade the neutralizing antibody response forcing the host to develop specific antibodies against these new epitopes in order to control viral replication (Montelaro et al., 1998a). While there is evidence that CTL responses also play a role in restricting EIAV replication (McGuire et al., 2004), there have been no studies investigating the effect of antigenic variation on these responses. Indeed, most studies investigating T cell responses in EIAV infected horses involve few or singular time points post-infection (Tagmyer et al., 2008a). Here we analyzed the dynamics of EIAV gp90 specific T cells responses over the first six months of the infection in ponies inoculated with the $EIAV_{D9}$ strain. We found that the T cell response undergoes expansion and contraction in terms of gp90 epitope recognition.

There was evidence of an overall reduction both in Vβ family utilization and the expression of IFNγ mRNA as the infection progressed. Exposure to viral antigen and cellular division are needed to maintain virus-specific T cells during chronic infection (Shin et al., 2007) and this dynamic shift in the immunodominant epitopes hierarchy in the newly infected horses may be the result of mutations in specific epitopes leading to an escape from T cell recognition. In the inapparent carrier, persistent recognition focusing on the more conserved peptides results in a more effective T cell response and virus replication is tightly controlled. However, it is also known that T cell escape mutations continue to

occur in chronic infections, albeit at a lower frequency (Geels et al., 2003). Thus, other factors might also account. In order to elicit T cell responses, a threshold number of antigen-specific T cells with a certain affinity within the T cell repertoire must be required. Studies found that T cells may undergo clonal exhaustion during persistent infections. The antigen-specific T cells may succumb to deletion, leading to the changes of T cells repertoire and epitope hierarchies. Spectratyping was used to identify the overall nature of the T cells' Vβ CDR3 repertoire involved in the peptide-specific responses (Lin and Welsh, 1998). We observed that not all Vβ families were represented, and within specific Vβ families there were both clonal and oligoclonal distributions of the Vβ CDR3 repertoire. Within each Vβ family, the predominant peak likely represented the dominant peptide-specific T cell clones with the smaller peaks representing other cells recruited to the site non-specifically. As expected, the breadth of T cell recruitment to the injection sites, as measured by Vβ CDR3 utilization, was correlated with the magnitude of IFNγ response early in the infection.

In our study, we also found that not all ponies showed gp90 specific cellular immune responses, although all ponies seroconverted one month post infection. This may have been due to our use of a live attenuated virus, whose replication is significantly decreased in horses (Craigo et al., 2005). This reduced viral replication may be less likely to trigger robust immune responses (Peng et al., 2005). Several of the ponies also exhibited elevated IFNγ production in the non-stimulated controls *in vitro* which could have been due to active cell-mediated immune responses against other EIAV proteins such as the gag or capsid proteins (Chung et al., 2004; McGuire et al., 1994b; Mealey et al., 2001a; Mealey et al., 2005b). Also, it is important to note that we were only detecting those peptides

present in the parental $EIAV_{D9}$ strain of the virus. The true breadth of the T cell response post-infection is likely much greater than we have detected. Likewise, the use of peptides based on the consensus sequence of HIV underestimates the total T cell response (Altfeld et al., 2003). Additionally, the peptides used in this study are 20 amino acids in length. Since the typical length of a T cell epitopes is around 8 to 10 amino acids, and we cannot exclude the possibility that our individual peptides might include multiple T cells epitopes. This could account for the high number of Vβ families involved in the specific peptide responses we measured.

In conclusion, our results indicate that T cell responses evolve during the early stage of EIAV infection. The interaction between virus mutation and T cell evolution needs to be further investigated.

4.6 Acknowledgements

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CHAPTER 5

Relationship between EIAV gp90 specific T cells recognition pattern and epitope variability in EIAV newly infected ponies

5.1 Summary

EIAV has been used as a model to investigate protective mechanisms against lentiviruses. Although studies have indicated that immune responses play an important role in controlling EIAV infection, the immune mechanisms that are responsible for this control are not yet identified. Since the virus undergoes rapid mutation following infection, the adaptive immune response must also evolve to meet this challenge. In our previous study, we found that gp90 specific T cells underwent expansion and contraction during the first six months after infection with an attenuated EIAV strain (Chapter 4). Here, we want to determine the relationship between EIAV gp90 specific T cell recognition and epitope variability in newly infected ponies. We hypothesized that gp90 specific recognition will shift from immunodominant variable determinants to conserved immunorecessive gp90 determinants following EIAV infection. Gp90 peptide-specific T cells responses were monitored *in vivo* over six months following EIAV infection. At the same time, approximately 87 virus clones from 25 ponies infected with $EIAV_{D9}$ for six months were analyzed. Finally, a new EIAV attenuated strain (EIAV ConD9) which contained a predicted consensus gp90 sequence was tested. Our results indicate gp90 epitope specific T cells target more variable regions of gp90 early post infection, whereas more conserved regions of gp90 were identified six months after infection.

5.2 Introduction

Equine infectious anemia virus (EIAV) has been used as a model for investigating the underlying protective immune mechanisms against lentiviruses (Craigo and Montelaro, 2011). There are three clinical stages after EIAV infection: acute, chronic, and inapparent carrier stages. One month post infection, most infected horses will develop an acute episode of disease characterized by fever, diarrhea, edema, lethargy, anemia, thrombocytopenia and viremia. During this acute phase, the immune system develops temporary control of the virus infection resulting in a decrease in virus load in the plasma. In the chronic phase of the infection, EIAV infected horses will show recurrent disease episodes. Sequencing of viruses isolated from the plasma during febrile episodes indicated that each occurrence of disease is caused by new viral quasispecies different from the previous episodes (Leroux et al., 1997; Zheng et al., 1997a). After 6-12 months, most horses control the EIAV infection and become inapparent carriers (Hammond et al., 2000; Harrold et al., 2000). Immune responses play an important role in controlling EIAV infection during this inapparent carrier stage (Mealey et al., 2001b; Perryman et al., 1988) as the administration of immunosuppressive drugs can induce the recurrence of clinical disease (Craigo et al., 2002a).

In a previous study, an attenuated vaccine strain $EIAV_{D9}$ was shown to provide protection against homologous virus challenges (Craigo et al., 2007b), but optimum protective immunity was not realized until six months post vaccination. Thus, protective immunity against EIAV infection is a lengthy process that likely involves the development of immunity to viral variants (Hammond et al., 2000). Analyses of longitudinal serum samples from EIAV infected horses identified an evolution of envelope-specific antibodies

responses as measured by changes in avidity, conformational recognition, and neutralization titers (Hammond et al., 1997; Hammond et al., 1999).

Cellular immune responses also appear important in controlling EIAV infections. The appearance of EIAV-specific cytotoxic T lymphocytes (CTL) correlates with the control of the initial viremia (McGuire et al., 2000). Most acute viral infections involve the early recognition of immunodominant epitopes leading to the eradication of the infection (Chisari and Ferrari, 1995; Perelson et al., 1993). By contrast, in chronic viral infections there is a shift in T cell recognition from immunodominant to subdominant epitopes (Fuller et al., 2004; Turner et al., 2005; Wherry et al., 2003). For example, initial HIV-specific T cell responses are skewed towards immunodominant epitopes in the antigenically variable regions of the virus in the initial stages of the infection, whereas subdominant epitopes in the more conserved regions are recognized during the later stages of infection (Goulder et al., 2001; Jamieson et al., 2003; Liu et al., 2013). Several studies indicated that protective immunity to HIV likely requires T cell recognition of these subdominant epitopes in the antigenically conserved regions of the virus (Frahm et al., 2006a; Goonetilleke et al., 2009) and the added possibility that different cellular immune responses might also exist between epitope-specific responses. In persistent virus infections, especially where antigens change overtime, immunodominance is largely influenced by the sequence and availability of peptide antigens.

In our previous study using the EIAV model, we found that peptide-specific T cell recognition patterns changed over the initial six months of the infection. By contrast, peptide recognition by a long-term inapparent carrier (D64) was more stable. Here, we want to determine the influence of antigen variation on the epitope recognition in EIAV

infected ponies. Virus clones from the ponies infected with the $EIAV_{D9}$ strain for six months were collected and the hypervariable regions of gp90 (V3 to V8) sequenced (Leroux et al., 2001; Leroux et al., 1997). Also, a new EIAV attenuated strain which contained a consensus gp90 sequence was used to determine the effect of concensus sequences on T cell epitope recognition and immunodominance.

5.3 Materials and Methods

5.3.1 Ponies, virus and experimental challenge

Sixteen ponies were intravenously injected with 10^3 $TCID_{50}$ of an attenuated $EIAV_{D9}$ strain (Craigo, Durkin et al. 2007). Also, a new live attenuated consensus (Con) $EIAV_{D9}$ strain constructed by insertion of a derived consensus gp90 into the backbone of $EIAV_{D9}$ virus was obtained from Dr. Ron Montelaro (Univ. of Pittsburgh). Eight ponies were infected with this newly developed Con $EIAV_{D9}$ strain virus. MHC haplotypes of the ponies were determined using polymorphic microsatellites as previously described (Tseng, Miller et al. 2010). For ponies receiving the attenuated $EIAV_{D9}$ strain, five ponies (G35, L132, H46, H38 and H32) shared the same MHC microsatellite allele 219-252-280-172-247, three of the ponies (L126, L132 and I28) shared the allele 211-260-268-166-247, and another three ponies (D49, H36 and H32) shared the same MHC microsatellite allele 211-260-278-174-241 (Table 5.1). For the ponies receiving Con D9 strain, only two ponies (G39 and G40) share the same MHC microsatellite allele 219-252-280-172-247 (Table 1).

All ponies were monitored for clinical symptoms of EIA throughout the course of the 6 months study. Rectal temperature was monitored daily and platelet numbers determined using an IDEXX QBC Vet Autoreader weekly. Plasma samples from ponies

receiving $EIAV_{D9}$ were collected weekly and stored at − 80ºC for later use to determine viral RNA per milliliter of plasma. Gag-specific amplification primers were designed and a quantitative real-time multiplex RT-PCR assay was used for analysis of viral RNA in plasma samples.

All animals were handled under the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture, according to protocols approved by the University of Kentucky Institutional Animal Care and Use Committee.

5.3.2 Determination of $EIAV_{D9}$ strain gp90 variation

Plasma was collected from each pony in the $EIAV_{D9}$ infected group six months post infection for virus sequencing. Virus sequencing was performed at the Department of Microbiology and Molecular Genetics, University of Pittsburgh. The cloning and sequencing of $EIAV_{D9}$ gp90 were conducted as previously described (Craigo et al., 2010). Approximately 87 $EIAV_{D9}$ gp90 clones from 25 ponies infected six months were analyzed. Multiple sequence alignments were conducted (http://www.genome.jp/ tools/clustalw/) and gp90 amino acid sequence variability was determined, as described (Bansal et al., 2005; Rodriguez et al., 2004). Normalized Shannon entropy scores were calculated for each amino acid position in the protein alignment using the program Scorecons (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl). Single peptide variability was calculated as the mean of normalized Shannon entropy scores for all amino acids located in the peptide.

Table 5.1 MHC haplotypes

Horse ID	Virus	UMN-JH34-2-COR112-COR113-UM011-COR114	UMN-JH34-2-COR112-COR113-UM011-COR114
G35	$EIAV_{D9}$	219-252-280-172-247	207-254-268-174-235
l126	$EIAV_{D9}$	211-260-268-166-247	211-260-268-166-247
L132	$EIAV_{D9}$	219-252-280-172-247	211-260-268-166-247
F31	$EIAV_{D9}$	205-252-274-168-243	203-260-266-168-249
I28	$EIAV_{D9}$	215-252-260-168-243	211-260-268-166-247
H46	$EIAV_{D9}$	221-268-278-174-241	219-252-280-172-247
D49	$EIAV_{D9}$	211-260-278-174-241	211-258-274-164-237
H38	$EIAV_{D9}$	219-252-280-172-247	223-252-268-166-247
H36	$EIAV_{D9}$	211-260-278-174-241	205-252-274-168-243
H32	$EIAV_{D9}$	211-260-278-174-241	219-252-280-172-247
D55	$EIAV_{D9}$	215-252-260-168-243	211-258-274-164-237
I35	$EIAV_{D9}$	205-262-270-184-245	221-262-270-172-237
J30	$EIAV_{D9}$	197-248-270-184-245	219-244-268-172-249
L124	$EIAV_{D9}$	221-262-270-172-237	221-238-264-180-243
I33	$EIAV_{D9}$	219-236-266-168-249	203-260-266-178-241
L128	$EIAV_{D9}$	219-250-266-170-245	211-262-270-184-245
G37	Con D9	205-252-274-168-243	205-262-264-170-239
G39	Con D9	219-252-280-172-247	209-254-268-178-251
G40	Con D9	203-260-266-168-249	219-252-280-172-247
I21	Con D9	215-264-270-172-249	219-238-266-178-241
I23	Con D9	205-262-270-184-245	215-262-272-168-255
I31	Con D9	197-248-270-184-245	219-236-266-168-249
K46	Con D9	UD*	UD*
L84	Con D9	221-268-278-174-241	207-262-272-168-255

*: Undetermined, Unable to find mare of K46, also could not find corresponding MHC alleles comparing with other existed known alleles

Table 5.2 Peptide sequence

EV0 peptide No.	EV0 peptide sequence	Con D9 peptide sequence
1	MVSIAFYGGIPGGISTPITQ	
2	PGGISTPITQQSEKSKCEEN	
3	QSEKSKCEENTMFQPYCYNN	
4	TMFQPYCYNNDSKNSMAESK	
5	DSKNSMAESKEARDQEMNLK	
6	EARDQEMNLKEESKEEKRRN	
7	EESKEEKRRNDWWKIGMFLL	
8	DWWKIGMFLLCLAGTTGGIL	
9	CLAGTTGGILWWYEGLPQQH	
10	WWYEGLPQQHYIGLVAIGGR	
11	YIGLVAIGGRLNGSGQSNAI	
12	LNGSGQSNAIECWGSFPGCR	
13	ECWGSFPGCRPFQNYFSYET	
14	PFQNYFSYETNRSMHMDNNT	PFQNYFSYETNRSMHIDNNT
15	NRSMHMDNNTATLLEAYHRE	NRSMHIDNNTATLLEAYHRE
16	ATLLEAYHREITFIYKSSCT	
17	ITFIYKSSCTDSDHCQEYQC	
18	DSDHCQEYQCKKVNLNSSDS	
19	KKVNLNSSDSSNPVRVEDVM	KKVNLNSSDSSIHVEDVED
20	SNPVRVEDVMNTTEYWGFKW	SIHVEDVEDTTEYWGFKW
21	NTTEYWGFKWLECNQTENFK	TTEYWGFKWLECNQTENFK
22	LECNQTENFKTILVPENEMV	
23	TILVPENEMVNINDTDTWIP	TILVPENEMVNINDNDTWIP
24	NINDTDTWIPKGCNETWARV	NINDNDTWIPKGCNETWARV
25	KGCNETWARVKRCPIDILYG	
26	KRCPIDILYGIHPIRLCVQP	
27	IHPIRLCVQPPFFLVQEKGI	
28	PFFLVQEKGIANTSRIGNCG	PFFLVQEKGIANTARIGNCG
29	ANTSRIGNCGPTIFLGVLED	ANTARIGNCGPTIFLGVLED
30	PTIFLGVLEDNKGVVRGNYT	
31	NKGVVRGNYTACNVSRLKIN	
32	ACNVSRLKINRKDYTGIYQV	
33	RKDYTGIYQVPIFYTCNFTN	
34	PIFYTCNFTNITSCNNEPII	PIFYTCNFTNITSCNNESII
35	ITSCNNEPIISVIMYETNQV	ITSCNNESIISVIMYETNQV
36	SVIMYETNQVQYLLCNNNNS	SVIMYETNQVQYLLCNNNNNS
37	QYLLCNNNNSNNYNCVVQSF	QYLLCNNNNNSNNYNCVVQSF

Table 5.2 (continued)

38	NNYNCVVQSFGVIGQAHLEL	
39	GVIGQAHLELPRPNKRIRNQ	
40	PRPNKRIRNQSFNQYNCSIN	PRPNKRIRNQNFNQYNCSIN
41	SFNQYNCSINNKTELETWKL	NFNQYNCSINNKTELETWKL
42	NKTELETWKLVKTSGITPLP	
43	VKTSGITPLPISSEANTGLI	
44	ISSEANTGLIRHKRDFGISA	

5.3.3 Production of synthetic peptides and construction of peptide pools matrix

Forty-four peptides, 20 amino acids in length, with 10 amino acids overlapping and spanning the entire gp90 of $EIAV_{D9}$ were synthesized by GenScript USA Inc. (Piscataway, NJ, USA) (Table 5.2). An additional 15 peptides specific (Table 5.2) for gp90 of Con D9 EIAV strain were also synthesized for the construction of a new consensus peptide pool matrix as shown in Figure 5.1. Each peptide was HPLC-purified, and the purity was above 85% confirmed by mass spectrometry. All peptides were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) at a stock concentration of 2 mg/ml. Peptides were suspended in 100ul saline for determining peptide-specific responses *in vivo*. The optimal working concentration for single peptides alone and within a peptide pool was determined using 3 fold titrations in horse D64. The optimum concentration was 5ug/ml.

A

Matrix of EV0 Peptides

	A	B	C	D	E	F	G
1	1	3	5	7	9	11	13
2	15	17	19	21	23	25	27
3	29	31	33	35	37	39	41
4	43	2	4	6	8	10	12
5	14	16	18	20	22	24	26
6	28	30	32	34	36	38	40
7	42	44					

B

Matrix of Con D9 Peptides

	A	B	C	D	E	F	G
1	1	3	5	7	9	11	13
2	Con15	17	Con19	Con21	Con23	25	27
3	Con29	31	33	Con35	Con37	39	Con41
4	43	2	4	6	8	10	12
5	Con14	16	18	Con20	22	Con24	26
6	Con28	30	32	Con34	Con36	38	Con40
7	42	44					

Figure 5.1 Peptide Matrix. A) Forty-four original autologous peptides from $EIAV_{D9}$ were synthesized. B) Fifteen consensus peptides were newly synthesized. In total, 14 peptides pools were constructed and each single peptide could be found in two different peptides pools.

5.3.4 Gp90-specific T cells responses *in vivo*

Our *in vivo* method for assessing immune reactivity to specific peptides, described in Chapter 3, was used to dissect the influence of virus variations on immune responses.

In general, all infected ponies were screened for gp90 specific cellular immune responses at 1, 3, and 6 months post infection. A 2mm skin biopsy was collected and homogenized for later RNA extraction. Total RNA was isolated using phenol: chloroform method (Chomczynski and Sacchi, 1987a). 1 µg RNA was dissolved in 41.5 µl nuclease-free water (Qiagen, Valencia, CA) and transcribed into cDNA, as previously described (Breathnach et al., 2006). The cDNA was stored in -20^{o}C until real-time PCR analysis was performed.

IFNγ gene expression was determined by real-time PCR, as previously described (Liu et al., 2011b). Equine-specific, intron-spanning TaqMan primers and probes were used for RT-PCR amplification. Amplification efficiencies were determined using Linreg (Ramakers et al., 2003) and only samples with amplification efficiencies above 99% were included for further analyses. Beta-glucuronidase (β-GUS) was used as housekeeping gene and the $^{\Delta\Delta}$CT method (Livak and Schmittgen, 2001b) was used to determine relative gene expression with saline injection site for each horse used as the calibrator. Relative quantity (RQ) was calculated as $2^{-\Delta\Delta CT}$.

5.3.5 Data Analysis

One way analysis of variance (one way ANOVA) was used to determine differences of normalized Shannon entropy scores over time. Unpaired t test was used to determine differences between the normalized Shannon entropy scores of early and later recognized peptides. Statistical significance was set at $p < 0.05$.

5.4. Results

5.4.1 Gp90-specific cellular immune responses in $EIAV_{D9}$ infected ponies *in vivo* over six months

In summary, 11 ponies showed gp90-specific responses following intradermal inoculations. One month post infection, 7 ponies exhibited gp90-specific T responses and, as shown in Figure 5.2, most recognized peptides were found in the carboxyl terminal region of gp90. After three months of infection, more peptides from the middle and amino terminal end of gp90 were recognized. At six months post infection, most ponies could recognize more than one peptide and these recognized peptides spanned the entire length of gp90.

5.4.2 Variability of gp90 in $EIAV_{D9}$ strain

Approximately 87 virus clones from the ponies infected with the $EIAV_{D9}$ strain for six months were collected and the hypervariable regions of gp90 (V3 to V8) sequenced (Leroux et al., 2001; Leroux et al., 1997). As shown in Figure 5.3A, the locations of the hypervariable regions of gp90 in $EIAV_{D9}$ were quite similar as those in other EIAV strains (Leroux et al., 1997). When the normalized Shannon entropy scores for each of the gp90 peptide was calculated (Figure 5.3B), the most variable peptides (#18, #19, #20) were found to be located within the V3 region which contains a known principal neutralizing domain (PND) (Leroux et al., 1997).

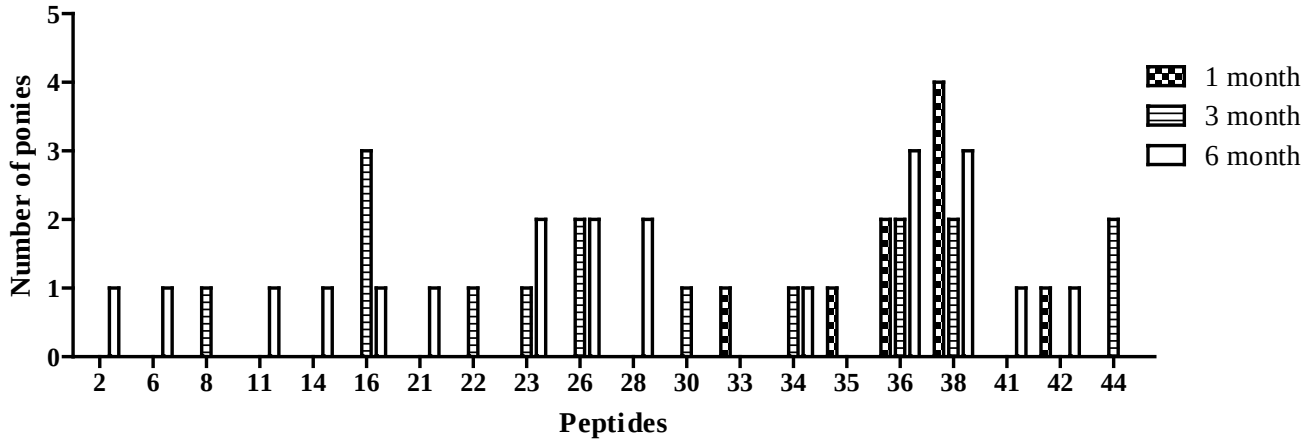
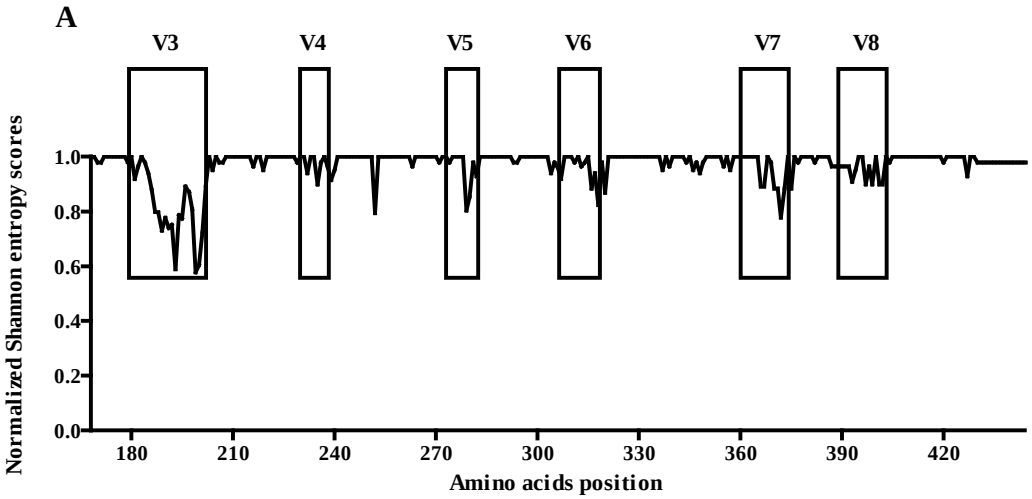


Figure 5.2 Summary of peptides recognition pattern in $EIAV_{D9}$ infected ponies over six months. X-axis indicates gp90 peptides which could elicit positive T cells responses during six months post infection. Y-axis indicated the number of ponies which could show gp90 positive responses.


A
V3
V4
V5
V6
V7
V8
Normalized Shannon entropy scores
1.0
0.8
0.6
0.4
0.2
0.0
180
210
240
270
300
330
360
390
420
Amino acids position

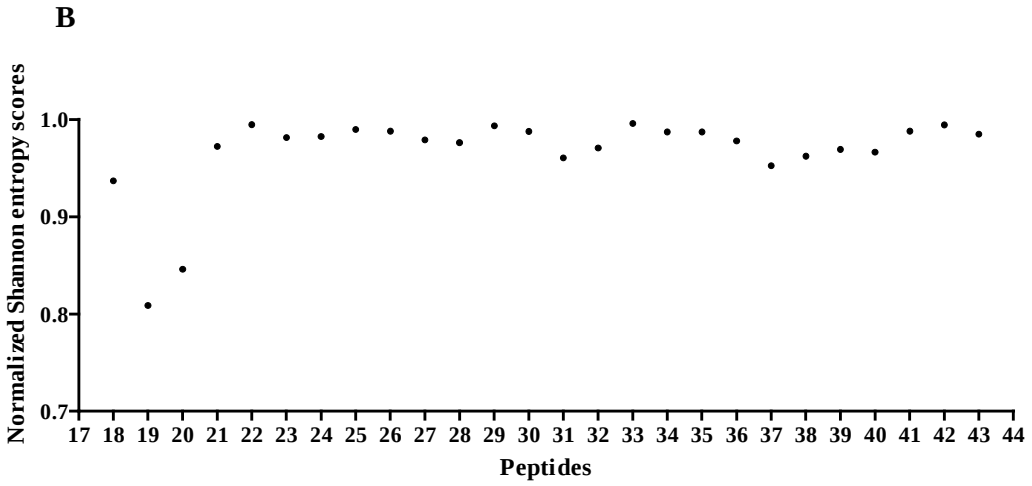

B
Normalized Shannon entropy scores
1.0
0.9
0.8
0.7
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44
Peptides

Figure 5.3 The relationship between peptides recognition pattern and the variability of peptides overtime. A) The variability of gp90 in the attenuated $EIAV_{D9}$ stain. X-axis indicates amino acids location. Y-axis indicated the normalized Shannon entropy scores. The lower the score, the more variable the regions are. The square boxes V3-V8 addressed the variable and conserved regions of gp90 identified in pathogenic EIAV stain (Leroux et al., 2001). B) Individual peptide variability. Y-axis indicated the average of the normalized Shannon entropy scores for each peptide.

5.4.3 Early recognized peptides were located in more variable regions of gp90

When the normalized Shannon entropy scores of newly recognized peptides among 1, 3, and 6 months post infection were compared (Figure 5.4A), results indicated the entropy scores of the newly recognized peptides in each time point increased over six months (p=0.07). One month post infection, the peptides recognized were located in more variable regions of gp90. In comparison, there were more peptides located in conserved regions were recognized at six months post infection. Having noticed that not all ponies showed gp90 specific immune responses in the first month post infection, we also separated peptides into first recognized and later recognized peptides for each pony. As indicated in Figure 5.4B, there was a significant increase of the normalized Shannon entropy scores between first recognized and later recognized peptides (p=0.03).

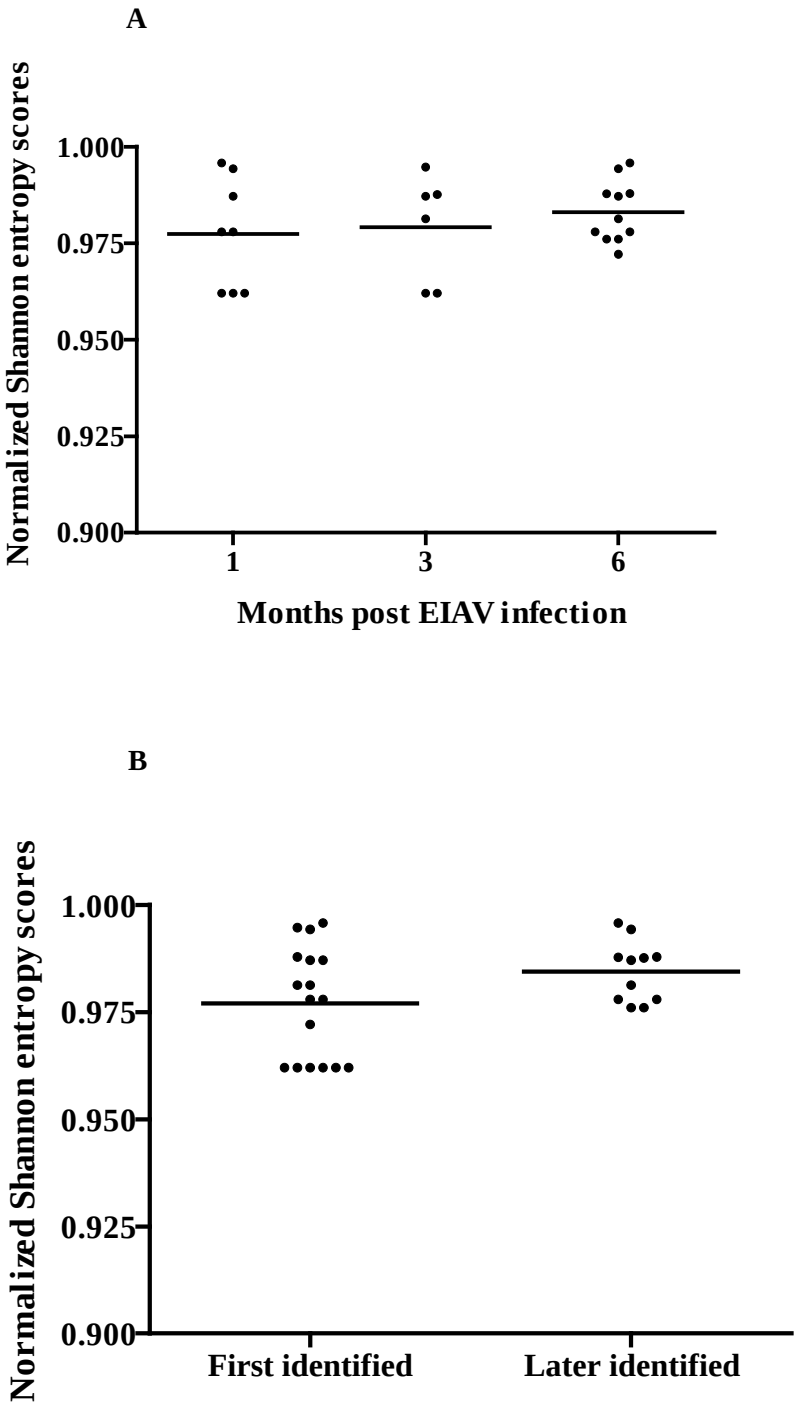

A
Normalized Shannon entropy scores
1.000
0.975
0.950
0.925
0.900
1
3
6
Months post EIAV infection
B
Normalized Shannon entropy scores
1.000
0.975
0.950
0.925
0.900
First identified
Later identified

Figure 5.4. Comparison of the normalized Shannon entropy scores overtime. A) Comparison of the normalized Shannon entropy scores among peptides recognized at 1, 3, and 6 months. One way analysis of variance (one way ANOVA) was used to determine differences of normalized Shannon entropy scores over time. B) Comparison of the normalized Shannon entropy scores between peptides recognized early and later in each pony. Unpaired t test was used to determine differences between the normalized Shannon entropy scores of early and later recognized peptides. Statistical significance was set at $p < 0.05$.

5.4.4 Gp90-specific cellular immune responses over time in newly constructed consensus D9 (Con D9) vaccinated ponies

As the majority of the peptides recognized were located in the variable regions of gp90 one month post EIAV infection and the more conserved peptides were recognized at later time points, it seems likely that the hypervariable region of gp90 might serve as an immune decoy. Deletion of these variable sequences might thus trigger T cells to recognize more conserved sequences earlier. To test this hypothesis, a new consensus D9 live attenuated EIAV strain was constructed and tested. Eight ponies were infected with this new EIAV strain, and gp90-specific cellular immune responses *in vivo* were determined at 1, 3, and 6 months post infection.

Each pony seroconverted by 35 days post infection, as determined by commercial ELISA kits (data not shown). There were no changes in rectal temperature or platelet counts in the ponies post-infection. At one month post infection, two ponies (K46 and L84)

showed positive gp90-specific cellular immune responses (Figure 5.5). All five peptides were located in section III of gp90, and four of five peptides were also located in the more variable regions of gp90. Six months post infection, more peptides located in sections I and II corresponding to conserved regions were recognized. Thus, peptide #16 located in a conserved was recognized by 3 ponies at six months post infection. Our results indicated that this newly constructed attenuated ConD9 strain could elicit similar immune responses as the attenuated $EIAV_{D9}$ strain.

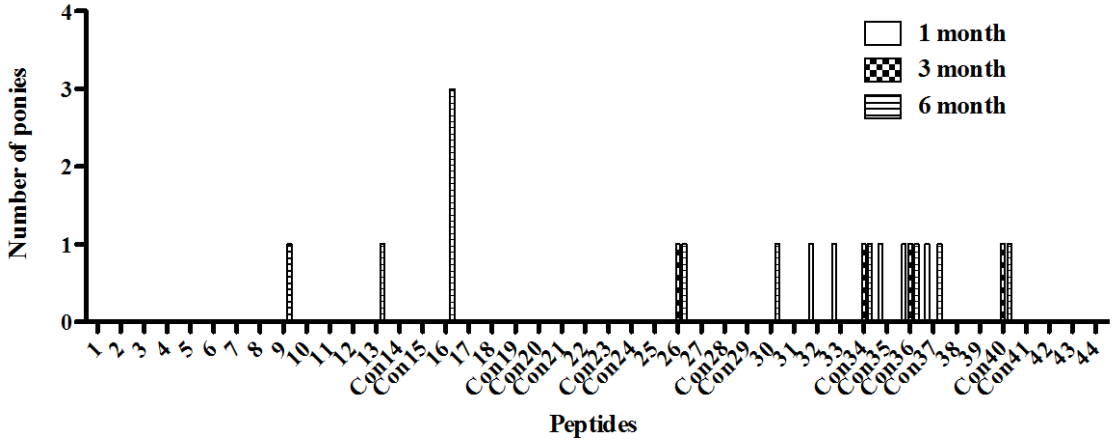


Figure 5.5 Peptide recognition pattern in EIAV ConD9 infected ponies over six months. Six ponies were infected with EIAV ConD9 stain, and CMI responses *in vivo* against gp90 was monitored. Y axis indicated the total number of ponies recognizing each peptide.

5.5 Discussion

The ability of lentiviruses to undergo antigenic variation, mainly in their envelope proteins, is the primary mechanism of immune evasion in the infected host (Burns and Desrosiers, 1994; Craigo and Montelaro, 2010; Payne et al., 1987). Previous investigations of antigenic variation in $EIAV_{D9}$ indicated that this virus also undergoes substantial mutation following infection with the median divergence being around 1.89% from the initial strain six months post inoculation (Craigo et al., 2010). During chronic infection, EIAV envelope gp90 also mutates such that distinct conserved and variable regions can be identified (Leroux et al., 2001; Leroux et al., 1997; Zheng et al., 1997a). In our study, we found that the $EIAV_{D9}$ strain undergoes similar changes in the conserved and variable segments gp90, similar to other pathogenic EIAV strains.

To deal with this high propensity for antigenic variation, immune responses must also adapt to the changing virus (Nowak et al., 1995a). In our previous study, we found that gp90 specific T cells underwent expansion and contraction during the first six months after $EIAV_{D9}$ infection (Chapter 4). In this chapter, we wanted to determine the relationship between EIAV gp90 specific T cells recognition pattern and epitope variability in EIAV infected ponies. We found that T cell responses changed continuously in terms of peptide recognition. Thus, one month post infection, most T cells targeted the variable regions of gp90. By six months post infection, T cells recognizing peptides located in the conserved regions emerged. Similar results were also found in an HIV study where HIV-specific T cell responses were skewed towards variable peptides in early HIV-1 infection, whereas more conserved peptides were recognized in chronic infections (Bansal et al., 2005; Goulder et al., 2001; Liu et al., 2011c). Virus mutations leading to the loss of the initial T

cell epitopes could be one of major reasons for the immunodominant recognition shifting from variable peptides to more conserved peptides. T cell responses exert strong selection pressure on the early recognized epitopes which have a greater tendency to mutate leading to an escape from T cell recognition (Cao et al., 2003; Kelleher et al., 2001; Mealey et al., 2004). Epitope entropy is thus correlated with the rate of the emergence of escape mutants. Using consensus peptides, some studies have shown that responses to variable regions diminish after acute infection and the disappearance of T cells responses was correlated with the appearance of these T cell escape mutants (Jamieson et al., 2003; Liu et al., 2011c).

Studies of HIV-specific T cells responses have shown that similar selection pressure causes escape mutations during the primary infection, but the virus escaped more slowly or was invariant in chronic infections (Goonetilleke et al., 2009). Our findings combined with others indicate that immunodominant epitopes recognized by the T cells during the acute phase of the infection may serve as immunodecoys, whereas immunorecessive epitopes may be more associated with long-term protection (Frahm et al., 2006a; van der Most et al., 1997). We also tested a newly constructed attenuated ConD9 strain which contain a consensus gp90 sequence. To our surprise, ponies receiving Con D9 strain showed similar gp90 specific T cells recognition pattern during the first six months post infection. Thus, gp90-specific T cells still recognized variable determinants one month post vaccination and showed dynamic shifting to the immunorecessive epitopes in the more conserved sequences six months post vaccination. Based on this finding, other factors may also influence the immunodominant recognition. For example, T-cell repertoire in the host can shape immunodominance as well. In order to elicit T cell responses, a threshold number of antigen-specific T cells with a certain affinity within the T cell repertoire must be

required. In chronic viral infection, studies found that T cell may undergo clone exhaustions during persistent infections. The antigen-specific T cells may succumb to deletion, leading to the changes of T cells repertoire and epitope hierarchies.

Importantly, our data did not show T cells exclusively targeting variable peptides during the acute phase of the infection, but rather an expansion of responses to the more conserved peptides during the chronic stage of the infection. When designing vaccines, it is important to know whether T cells recognition differs between acute and chronic infection. If so, it is vital to determine which responses should be generated and incorporated within a candidate vaccine.

5.6 Acknowledgements

The authors wish to thank all farm staff for their technical support and care of all ponies. This work was supported by NIH R01 AI25850-23.

CHAPTER 6

Conclusion and future directions

6.1 Conclusion

More than 30 million people are infected with HIV, with the estimated AIDS-related deaths around 1.8 million people in 2009. The development of a successful AIDS vaccine is urgently needed to protect and treat HIV infection. However, there are many challenges to develop a successful AIDS vaccine. Vaccines are a potent tool to prevent and treat different infectious diseases (Pantaleo and Koup 2004). One reason for the failure of AIDS vaccines is that the nature of an effective immune response that correlates with protection is unknown (Klausner, Fauci et al. 2003; Pantaleo and Koup 2004). The complexities of AIDS vaccine research, coupled with the lack of a successful human AIDS vaccine, accentuate the need for animal models to investigate the protective immune mechanisms against lentiviral infection.

Identified in 1843, EIAV was later classified as a lentivirus (Craigo and Montelaro 2011). Distinct from other lentiviruses, EIAV infection can be controlled by the horses' immune system. This naturally gained protective immunity makes EIAV a good model for investigating the underlying protective immune mechanisms against lentivirus. It has also been shown that an attenuated virus vaccine ($EIAV_{D9}$) could provide 100% protection against homologous virus challenge (Li, Craigo et al. 2003; Tagmyer, Craigo et al. 2007), such that this attenuated virus vaccine could serve as a model to investigate the virus-specific immune responses associated with the enduring broadly protective immunity.

Up till now, the greatest challenge for the development of an effective HIV vaccine might be the envelope antigenic variation and associated immune evasion of lentiviruses. Work on EIAV has shown that envelope variation is a primary mechanism responsible for immune evasion such that even minor changes in envelope protein sequences can markedly affect recognition and protective immunity (Craigo, Zhang et al. 2007). To face this high propensity for antigenic variation, we believe that immune responses also evolve (Nowak et al., 1995a). The major focus of this dissertation is to evaluate the changes of cellular immunity in EIAV infected ponies, and the influence of gp90 antigenic variation on cellular immune recognition. Using a new established *in vivo* assay, we found that gp90-specific T cell responses underwent expansion and contraction during the first six months post $EIAV_{D9}$ infection. Further analysis of the influence of sequence variations on T cells recognition indicated that T cells recognition was skewed towards variable peptides in early EIAV infection, whereas towards more conserved peptides after 6 months infection. In contrast, the peptide recognition pattern in an inapparent carrier was more stable over three years.

Comparison of the MHC haplotypes showed that only L132 and H46, D49 and H36 shared the same haplotypes. The major length of T cells epitopes are usually around 8 to 10 amino acids, and we could not exclude the possibility that our peptides (20 amino acids length) might include multiple T cells epitopes. Also, broadly recognized T cells epitopes are detected in EIAV infected ponies (Chung et al., 2004), ponies with different MHC haplotypes may also recognize the same epitopes. Our finding indicated that cellular immune responses in EIAV infected ponies undergo a gradual maturation during the early infection, and the stable recognition in the apparent carrier indicates an enduring protective

immunity in the chronic infection stage. The epitope shifting from variable to conserved regions implies that T cells recognizing conserved epitopes are more important in controlling EIAV infection. When designing vaccines, it is important to know whether the T cells recognitions differ between acute and chronic infection. If so, it is vital to determine which responses should be generated and incorporated within a candidate vaccine. Based on our results and others, it seems that immune responses targeting subdominant conserved regions are more critical component against lentiviral infections (Frahm et al., 2006b).

6.2 Future direction

Understanding the mechanisms that may influence this immunodominant and immunodominant shifting will provide useful information for vaccine development. There are two major parameters that may shape the immunodominance: antigen-related factors and T cell-related factors. In persistent viral infections, especially like HIV which antigens change overtime, immunodominance is largely influenced by the sequence and availability of peptide antigens. Mutations in the anchoring residues may significantly reduce the affinity and stability of peptides binding with the MHC molecule. In this situation, the presentation of the mutated peptide antigens to T cells may be diminished or out-competed by other subdominant peptides. T cells responses exerted strong selection pressure on the early recognized epitopes and the selected epitopes have a high propensity to mutate and result in T cells escape (Cao et al., 2003; Kelleher et al., 2001; Mealey et al., 2004). Although T cell escape has been identified in EIAV infected horses in the previous study, the interaction between T cells escape mutant and T cells recognition pattern changes need to be further studied.

Also, it appears that the T-cell repertoire in the host can shape the immunodominance as well. In order to elicit T cell responses, a threshold number of antigen-specific T cells with a certain affinity within the T cell repertoire must be required. In chronic viral infection, studies found that T cell may undergo clone exhaustions during persistent infections. The antigen-specific T cells may succumb to deletion, leading to the changes of T cells repertoire and epitope hierarchies. Also, peptide specific T cells detected early have high affinity toward the epitopes. These T cells with high affinity could compete with other T cells and apply selection pressure on the virus (Lichterfeld et al., 2007; Mealey et al., 2003a; Nowak et al., 1995b). In our study, we found that the epitope specific T cells repertoire correlates with the magnitude of T cells responses *in vivo* in two ponies. Whether the T cells receptor repertoire and T cells affinity have any correlation with this immundominant recognition and immunodominant shifting need to be further investigated.

APPENDIX 1

Granzyme B-mRNA expression by equine lymphokine activated killer (LAK) cells is associated with the induction of apoptosis in target cells

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Abstract

Lymphokine-activated killer (LAK) cells are a subset of cytotoxic cells capable of lysing freshly isolated tumor cells. While LAK activity is typically measured using the ^{51}Cr-release assay, here we used a non-radioactive flow cytometric method to demonstrate equine LAK activity. Equine peripheral blood mononuclear cells (PBMC) were stimulated *in vitro* with recombinant human interleukin 2 (hIL-2) to generate LAK cells. An equine tumor cell line, EqT8888, labeled with carboxyfluorescein succinimidyl ester (CFSE) was used as target cells. Following incubation of the targets with different concentrations of LAK cells, Annexin V was added to identify the early apoptotic cells. With increasing effector to target cell ratios, EqT8888 apoptosis was increased. We also measured interferon-gamma, granzyme B and perforin mRNA expression in the LAK cell cultures as possible surrogate markers for cytotoxic cell activity and found granzyme B mRNA expression correlated best with LAK activity. Also, we found that the reduced LAK activity of young horses was associated with decreased granzyme B mRNA expression. Our results indicate that fluorescence-based detection of LAK cell activity provides a suitable non-radioactive alternative to ^{51}Cr-release assays and mRNA expression of granzyme B can be used as surrogate marker for these cytotoxic cells.

Keywords: equine LAK cells; flow cytometric assay; granzyme B; perforin; IFNγ; real-time PCR

1. Introduction

Cell-mediated cytotoxicity (CMC) is an important effector mechanism directed against transformed and infected cells. Natural killer (NK), lymphokine activated killer (LAK) and cytotoxic T lymphocytes (CTL) are examples of these effector cells. The granules of cytotoxic lymphocytes contain potent mediators which include pore-forming protein (perforin), cytotoxic cytokines, and granzymes (a family of serine esterases). Together these mediators can induce death of the target cells via caspase-dependent and independent apoptotic pathways (Young et al., 1988). While the ^{51}Cr release assay has been widely used to demonstrate CMC (Brunner et al., 1968), this assay has several disadvantages including the use of a radioactive isotope, high levels of spontaneous release by some targets, and a failure to identify the mechanism of cytotoxicity. Since the ^{51}Cr-release assay only detects the late phase of cytotoxicity, it does not distinguish between apoptosis and other mechanisms of cell lysis. Alternative methods using non-radioactive approaches can overcome some of these limitations.

Flow-cytometric methods have been developed which use fluorescent regents to detect target cell killing either as an increase in membrane permeability (Flieger et al., 1995)or by changes in membrane morphology associated with apoptosis (Goldberg et al., 1999). In addition to avoiding the use of radioactive reagents, this approach offers overall greater sensitivity than the ^{51}Cr-release assays (Aubry et al., 1999) and allows for detection of both early and late phases of cell killing (Vermes et al., 1995).

An alternative approach to quantitating CMC is the direct detection of the cytotoxic cell population using antibodies either against specific cell surface proteins (Kim et al., 2007) or the effector molecules involved in target cell lysis. While cell surface staining can identify cells with cytolytic potential (*e.g.* NK, $CD8^+$ T cells, etc), it fails to detect effector function. Detection of specific effector molecules has been used to identify functional cytotoxic cells. Interferon-gamma (IFNγ) has frequently been used in equine research as a surrogate marker for cytotoxic cell activity (Breathnach et al., 2005; Ellis et al., 1997; Paillot et al., 2006). However, studies in other species have shown that IFNγ production does not correlate well with cytotoxic activity (Calarota et al., 2006). Given these findings and the fact that non-cytolytic $CD4^+$ T cells can also produce IFNγ, we believe better surrogate markers for equine cytotoxic effector function are needed. Granzyme B and perforin, two major effector molecules in the granule-mediated cytolytic pathways, are better markers since studies have shown that their expression correlate well with cytotoxic activity in PBMC cultures (Shafer-Weaver et al., 2003; Zuber et al., 2005)

In this report, we use a flow cytometric method for the characterization of equine LAK cell-mediated cytotoxicity using CFSE-labeled target cells and PE-Annexin V to identify target cells in the early apoptotic phase. We also examined three surrogate markers for LAK activity; IFNγ, granzyme B and perforin mRNA expression and found that granzyme B mRNA expression correlates best with cytotoxic activity of LAK cells.

2. Materials and method

2.1. Horses

Five 10 year old horses were used for the initial optimization of the flow cytometric and gene expression assays. Five additional 1 year old mix-breed horses were used to compare LAK activity and gene expression in the different aged horses. All horses were owned by the Department of Veterinary Science, University of Kentucky and handled in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture. All research procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.2. Target cells

Equine tumor cell line, EqT8888 (Hormanski et al., 1992), was used as target cells in the flow cytometric assay. The cells were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM glutamine (Sigma), and 100U/ml penicillin/streptomycin (Sigma) at 37 ^{o}C 5% CO_2 in air. Target cells were harvested in the log phase of growth before CFSE staining.

2.3. LAK cell generation

Heparinized blood was collected via aspetic venipuncture of the jugular vein. Peripheral blood mononuclear cells (PBMC) were isolated using Ficol-Paque Plus^{TM} (Amersham Biosciences, Piscataway, NJ) gradient centrifugation according to the manufacturer's protocol. The PBMC were washed with phosphate-buffered saline (PBS) for three times before re-suspending at $3X10^6$ cells/ml in RPMI-1640 (Gibco, Grand Island, NY)

supplemented with 2.5% (v/v) fetal equine serum (FES, Sigma, St.Louis, MO), 2mM glutamine (Sigma), 100U/ml penicillin/streptomycin (Sigma), and 55 µM 2-mercaptoethanol (GIBCO, Grand Island, NY). The PBMC were incubated up to 5 days in T25 flasks supplemented with different concentration of recombinant human interleukin 2 (hIL-2; R&D Systems, Minneapolis, MN) to generate LAK cells (Hormanski et al., 1992). All cultures were incubated at 37^{O} C in 5% CO_2 incubator. Each day, the LAK cells were harvested and counted using a ViCell-XR instrument (Beckman Coulter, Miami, FL) before use.

2.4. LAK cell flow cytometric assay

EqT8888 was re-suspended at 1.0×10^7 cells/ml in 1ml PBS followed by the addition of 1ml solution of 3 µM CFSE (Fluka BioChemika, Buchs, Switzerland). The solution was gently mixed for exactly 8 min followed by the addition of 2ml of FBS to stop the reaction. The target cells were washed three times with 10% (v/v) FBS in PBS and re-suspended at a concentration of $3X10^5$ cells/ ml prior to dispensing 100 µl of labeled cells into a 96 well round bottom plate (Corning, Corning, NY). To each well of target cells was added a 100 µl volume of LAK cells at different cell concentrations to yield effector: target (E: T) ratios of 40, 20, 10 and 5:1. Plates were then centrifuged at 200g for 5 min and incubated at 37ºC in 5% CO_2 incubator. Samples with target cells only were used as spontaneous apoptosis controls. To generate apoptosis positive control samples, EqT8888 cells were incubated for 2 h in a 56ºC water bath. Killing of the cells was confirmed by trypan blue staining. All samples were performed in triplicate.

After 3 h incubation, the cells were transferred to 96 well V bottom plates. The plates were centrifuged at 300g for 5 min. Cells were washed by PBS for 2 times and re-suspended in 100 µl annexin V buffer (BD Pharmingen™), and transferred to 12 x 75mm tubes (BD Pharmingen™). Annexin V (PE-conjugated, BD Pharmingen™) was added to each tube and the cells were incubated in the dark at room temperature. After 15 min incubation, all samples were acquired using a FACSCalibur and analyzed by CELL Quest Pro™ (Becton Dickinson).

The target cells were gated using FL1 and 3000 gated events were acquired for each sample. Target cells that underwent apoptosis were both CFSE (FL1) and Annexin V-PE (FL2) positive. Each determination was performed in triplicate. The percentage of cytotoxicity was determined by the equation % cytotoxicity=

$$\frac{(\%CFSE^{+}Annexin\ V^{+}\ target\ in\ test\ well)-(\%CFSE^{+}Annexin\ V^{+}\ spontaneous\ release\ group)}{(\%CFSE^{+}Annexin\ V^{+}\ total\ release\ group)-(\%CFSE^{+}Annexin\ V^{+}\ spontaneous\ release\ group)} \times 100$$

2.5. Relative quantitation of granzyme B, perforin and IFNγ gene expression by real-time PCR

Approximately $3X10^{6}$ LAK cells from each horse were collected and re-suspended in 1ml RNA-STAT 60 (Tel-Test, Friendswood, TX) at the same time as the LAK cell flow cytometric assay was performed. Total RNA was extracted using a phenol: chloroform method (Chomczynski and Sacchi, 1987b). 1 µg RNA was dissolved in 41.5µl nuclease-free water (Qiagen, Valencia, CA) and transcribed into cDNA using 38.5µl reverse transcription master mix (1 µl oligo dT primer [0.5 µg/ml; Promega], 4 µl dNTP [10mM;

Promega], 0.5 µl avian myeloblastosis virus [AMV] reverse transcriptase [20U/ml; Promega, Madison, WI], 16 µl AMV buffer [Promega], 1 µl RNAsin [40U/ul, Promega], and 16 µl $MgCl_2$ [25mM, Promega]), as previously described (Coombs et al., 2006). The reactions were incubated at 42°C for 15 min and then 95 ℃ for 5min. The cDNA was stored in -20°C until real-time PCR was performed.

Granzyme B, perforin and IFNγ (Coombs et al., 2006) gene expressions were determined by real-time PCR (ABI systems 7900 Real-Time PCR Instrument, Foster City, CA). All primer/probe sets were designed in intron-spanning region and failed to amplify genomic DNA and reverse transcription negative RNA samples. The primer/probe sequences were shown in Table 1. PCR reactions were incubated in duplicate wells for 95°C 10 min, followed by 40 cycles of 95 °C for 15s and 60 ℃ for 60s. Each reaction contains 4.5 µl cDNA and 5.5 µl master mix (0.5 µl 20X assay mix for primer/probe set of interest [Applied Biosystems], and 5 µl TaqMan™ [Applied Biosystems, Foster City, CA]). Internal PCR amplification efficiencies were calculated in LinReg PCR (Ramakers et al., 2003) to confirm amplification efficiencies were above 99%. For relative quantitation, the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001b) was used to determine changes in gene expression. Beta-glucuronidase (β-GUS) was used as the housekeeping gene. The calibrator for each target gene was calculated using the average of the medium control samples of each horse. Relative quantity (RQ) was calculated as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001b).

2.6. Statistical analysis

All data were analyzed for statistical significance using SigmaStat (Sigma Stat version 3.5; Systat, San Jose, CA). One way analysis of variance (One way ANOVA) was used to determine differences over time (1-5 days). Two way analysis of variance (Two way RM ANOVA) was used to determine differences between separate age groups over time (1-5 days). Holm-Sidak method was used to identify differences in LAK activity and gene expression over time and between horses of different ages. Statistical significance was set at $p<0.05$. Correlations among granzyme B, perforin, IFNγ mRNA expression and flow cytometric assay results were determined using the Pearson Product Moment Correlation. Variables with positive correlation coefficient (r) and *p* values below 0.05 increased together whereas variables with negative correlation coefficient (r) were inversely correlated. There was no significant relationship when $p>0.05$.

3. Results

3.1. Target cell labeling with CFSE

One of the basic requirements for the flow cytometric determination of cytotoxicity is stable labeling of target cells. Figure 1 shows the stability of the CFSE-labeled EqT8888 cells at 1 h, 2 h and 3 h after staining. The mean fluorescence of CFSE did not change over the 3 h incubation period (data not shown).

3.2 Demonstration of LAK cell activity

Equine PBMC were incubated with 200 units/ml recombinant hIL-2 for 96 h to generate LAK cells. The LAK cells were added to labeled EqT8888 target cells to yield final effector: target (E:T) ratios of 5, 10, 20 and 40. As the E: T ratio increased, the percentage of $CFSE^+$ Annexin V^+ target cells also increased (Fig. 2a). Similar results were obtained when five different horses were used as the source of PBMC to generate LAK activity, though the overall cytotoxic activity for each individual horse was different (Fig. 2b).

3.3 Equine LAK activity is dependent on the doses of recombinant hIL-2

The generation of equine LAK activity is dependent upon the concentration of hIL-2 in the culture (Hormanski et al., 1992). The PBMC from 5 horses were incubated with different doses of recombinant hIL-2 for 96 h to generate LAK cells and assayed for the induction of apoptosis in the EqT8888 cells (Fig. 3). The ability of the LAK cells to induce apoptosis in the target cells was dependent on the doses of recombinant hIL-2 added to the LAK cell cultures. The dose required for optimal killing was also dependent upon the horse tested.

3.4 Temporal induction of Equine LAK activity

PBMC were cultured with 2000 units/ml recombinant hIL-2 for 5 days. On each day, cells were collected and tested for LAK activity using the flow cytometric assay for target cell apoptosis. As shown in Figure 4a, LAK activity increased significantly with incubation time ($p<0.001$) and optimal killing was obtained after 4 days incubation.

3.5 Comparison of granzyme B, perforin and IFNγ mRNA expression with cytotoxic activity in equine LAK cell cultures

Perforin, granzyme B and IFNγ have been used as surrogate markers for equine cytotoxic cells (Issel et al., 1992; Paillot et al., 2005; Patton et al., 2005; Viveiros and Antczak, 1999). Here, we determined mRNA expression of these markers in LAK cell cultures. PBMC were collected from five horses and cultured with 2000 units/ml recombinant hIL-2 for 5 days, as above. Each day, cells were collected for real-time PCR analysis of mRNA expression. Incubation with hIL-2 was associated with the increased expression of mRNA for granzyme B, perforin and IFNγ though with different kinetics. Similar to LAK activity, granzyme B mRNA expression increased with time (p=0.014). However, IFNγ mRNA expression markedly increased only during the first day of culture (p<0.001) and decreased significantly (p<0.001) thereafter. Perforin mRNA expression was induced by hIL-2 treatment, but remained fairly stable throughout the culture period (Figure 4a). When we determined the relationship between gene expression and LAK activity, there was an only significant positive correlation between EqT8888 apoptosis and granzyme B mRNA expression (Figure 4b).

3.6 Reduced LAK activity in young horses is associated with decreased granzyme B mRNA expression

PBMC from 1 year and 10 year old horses were cultured with 2000 units/ml human recombinant IL-2 for 5 days for both flow cytometric assay and real-time PCR. As shown in Figure 5, the 1-year-old horses' LAK activity was significantly (p<0.001) lower than the 10-year-old group over incubation time. This reduced cytotoxic activity was associated

with significantly ($p<0.001$) lower expression of mRNA for granzyme B (Figure 5), but not perforin or IFNγ (data not shown).

Discussion

Cytotoxic cells play an important role in cell mediated immune responses against tumors and intracellular pathogens. The most frequently used and widely accepted method for determining cell-mediated cytotoxicity *in vitro* is the ^{51}Cr release assay. While this assay is sensitive and repeatable, it requires the use of radioactive materials and is time-consuming to perform. Additionally, the ^{51}Cr release assay cannot determine the ultimate mechanism of cellular killing: cytolysis or apoptosis. There are two mechanisms whereby cytotoxic cells induce apoptosis in their targets: granule exocytosis and Fas-FasL pathway. Both NK and LAK cells have been shown to use the granule exocytosis pathway (Goldberg et al., 1999; Henkart and Sitkovsky, 1994). In this pathway, granzyme B enters the target cells via pores formed by perforin and cleaves procaspase-3 leading to the activation of the caspase cascade (Derby et al., 2001). Once the caspases are activated, DNA fragmentation and apoptosis occur (Henkart and Sitkovsky, 1994). Apoptosis is associated with the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer which can be detected using annexin V (Vermes et al., 1995). We have previously shown that incubation of equine PBMC with hIL-2 induces LAK activity, as measured by ^{51}Cr-release from EqT8888 cells (Hormanski et al., 1992). Here, we have utilized a non-radioactive flow cytometric assay to identify phosphatidylserine residues on the plasma membrane of the EqT8888 target cells following incubation with equine LAK cells. As such, our results indicate that equine LAK cells induce target lysis via the induction of an apoptotic pathway.

Consistent with equine LAK cells using a granule-mediated pathway, we observed a correlation between cytotoxic activity against EqT8888 cells and granzyme B mRNA expression in the hIL-2 stimulated PBMC cultures. A similar relationship between human LAK activity and granzyme expression has been reported (Lowrey et al., 1988). Perforin mRNA expression was increased with IL-2 stimulation, though not correlated with cytotoxic activity. Again, these results are consistent with previous reports on human NK/LAK induction where perforin induction was induced by IL-2 stimulation, but not necessarily correlated with cytotoxic activity (Clement et al., 1990). As both proteins exist as preformed mediators in the granules of cytotoxic effector cells, it is perhaps not surprising that there was not a better correlation between their expression and absolute cytotoxic activity. This also likely reflects the multiple steps involved in cytotoxic activity including recognition and binding of the target cells, formation of the synapse, lysis of target cells, and recycling of the effector cells.

In addition to their cytotoxic activity, LAK cells also secrete inflammatory cytokines such as IFNγ(Choi et al., 2008). Here, too, we detected increased expression of this cytokine post-IL-2 stimulation. In contrast to the other markers, IFNγ expression peaked early in the culture period and was thus inversely correlated with LAK activity in the culture over time. While IFNγ has frequently been used as surrogate marker for cytotoxic cell activity in equine research (Breathnach et al., 2005; Ellis et al., 1997; Paillot et al., 2006), this lack of correlation with cytotoxic activity and the fact that other non-cytotoxic cells produce this cytokine, makes it a poor choice as a surrogate marker for LAK cells.

While several studies of human LAK activity have shown similar levels of cytotoxicty in PBMC cultures from young children compared to adults (Ayello et al., 2009; Montagna et al., 1988), we observed a significant difference in cytotoxicity and granzyme B gene expression between the young and older horses used in this study. While the reasons for this age-dependent difference in the horse are unknown, one possible explanation could be the sources of the LAK activity. Human LAK activity is associated with both activated NK cells as well as $CD8^+$ TcR^+ cells (Mingari et al., 1987; Provinciali et al., 1995), these latter cells likely representing CTL. In the horse, LAK activity is similarly associated with a population of $CD3^+$ $CD8^+$ cells (Lunn et al., 1996; Viveiros and Antczak, 1999) and equine NK cells do not exhibit cytotoxic activity against the EqT8888 cell line (Viveiros and Antczak, 1999). Younger horses likely have a lower frequency of CTLs in their peripheral blood compared to adults which could account for their reduced LAK activity.

In summary, we have utilized a flow cytometric assay to demonstrate equine LAK cell activity. In addition to providing several advantages in terms of safety and cost, this approach allowed for the identification of apoptosis as the likely killing mechanism. RT-PCR analysis of IL-2 stimulated gene expression in LAK cell cultures provided additional supporting evidence for a granule-mediated cytotoxicity involving perforin and granzyme B, but not IFNγ. We were also able to determine that the reduced LAK activity in young horses was associated with decreased expression of granzyme B mRNA, likely reflecting fewer LAK precursor cells in their circulation.

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Table 1. Primer/probe sets used for gene expression measurements

Gene	Sequence (5'-3')		Amplicon length (bp)
β-GUS	Forward	GCTCATCTGGAACTTTGCTGATTTT	85
	Reverse	CTGACGAGTGAAGATCCCCTTT	
	Probe	CTCTCTGCGGTGACTGG	
IFNγ	Forward	AGCAGCACCAGCAAGCT	75
	Reverse	TTTGCGCTGGACCTTCAGA	
	Probe	ATTCAGATTCCGGTAAATGA	
Granzyme B	Forward	GGACCCGAAGGAAAAGAAGTCTT	74
	Reverse	CCTGGATCACGTTCTTACACACAAG	
	Probe	CCGGAGTCCCCCTTAAA	
Perforin	Forward	GCTTCAGCAGCGACTCAGT	54
	Reverse	CGTGCACCAGGCGAAA	
	Probe	ACTGTAGAAGCGACACTCC	

β-GUS=beta-glucuronidase; Probe: dual-labeled oligonucleotide with 5-FAM and 3-NFQ;
β-GUS, IFNγ (Coombs et al., 2006).

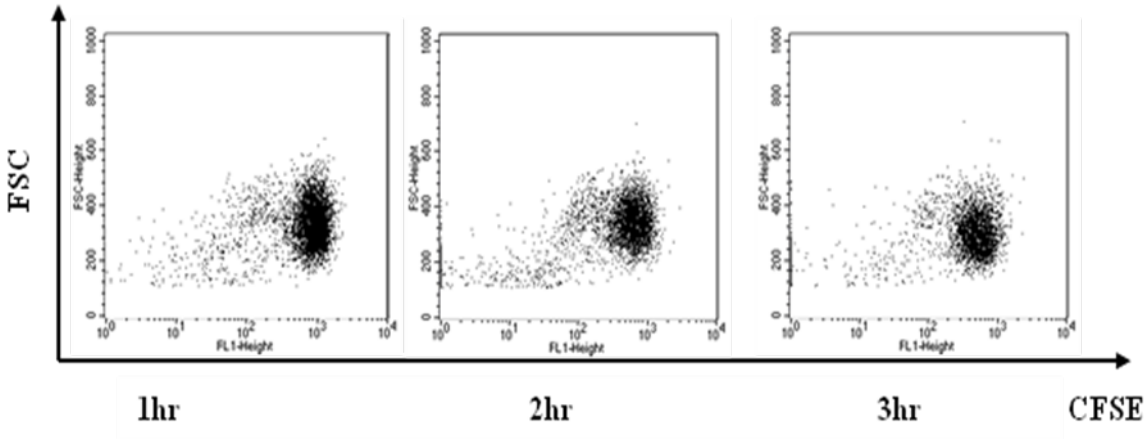
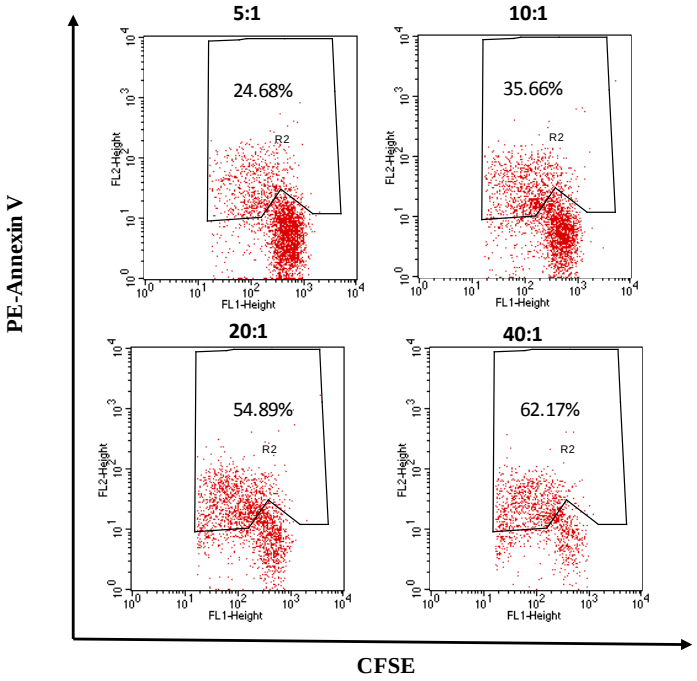


Figure 1. EqT8888 cells exhibit stable labeling with CFSE. 1.5µM CFSE was added to $7X10^6$ EqT8888 cells. After 1h, 2h and 3h incubation, EqT8888 cells were acquired and analyzed using flow cytometry. Data is representative of multiple experiments.

(a)



(b)

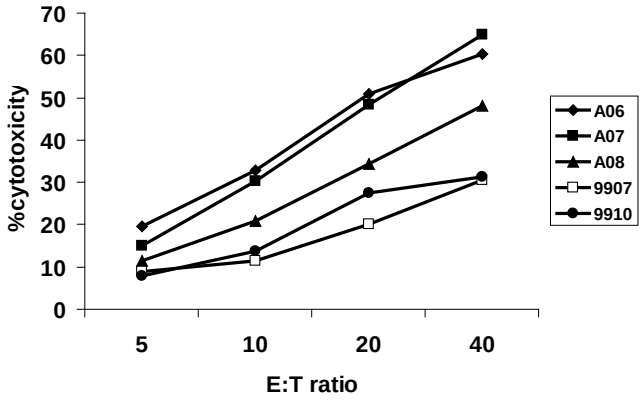


Figure 2 Flow cytometirc detection of equine LAK activity. (a) Apoptosis induction in EqT8888 cells by equine LAK cells. 30,000 target cells were cultured for 3 h in 5% CO_2 with different number of LAK cells yielding E: T ratio from 5, 10, 20 to 40. Data from (A06) is shown. (b) LAK cell activity of multiple horses. While the percentage of apoptotic target cells increased as the E:T ratios increased in all cases, overall LAK activity was different among the measured. All determinations were performed in triplicate.

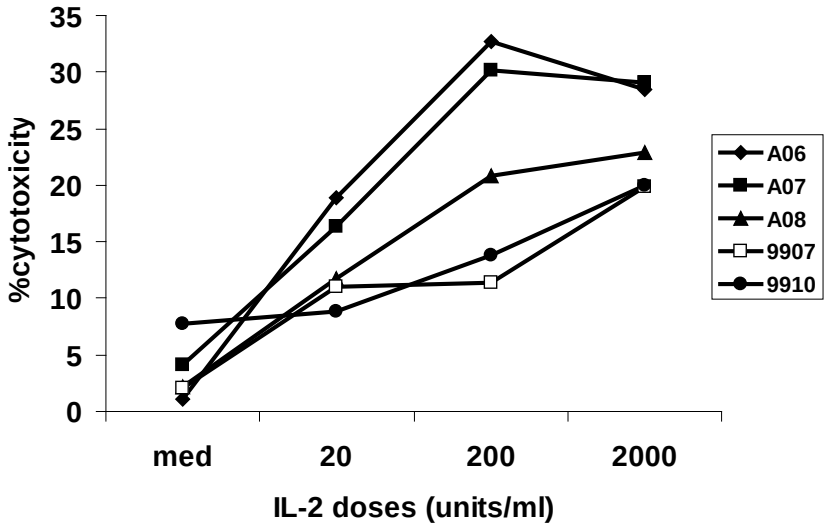
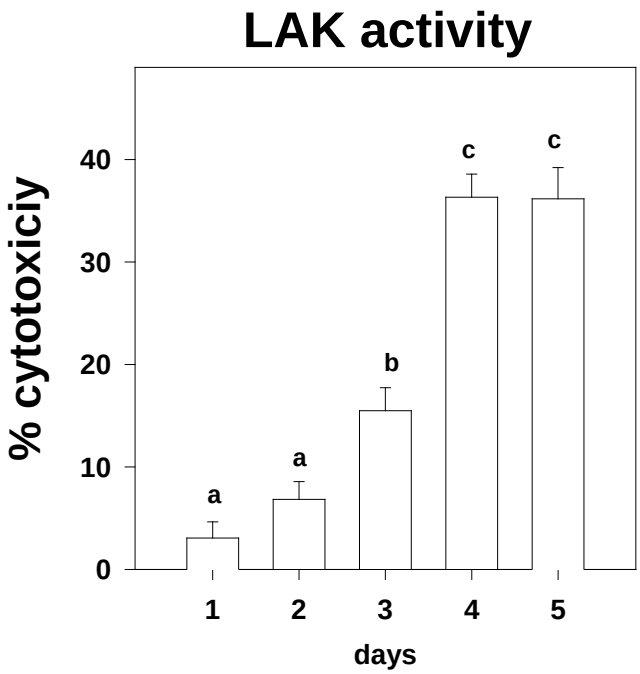
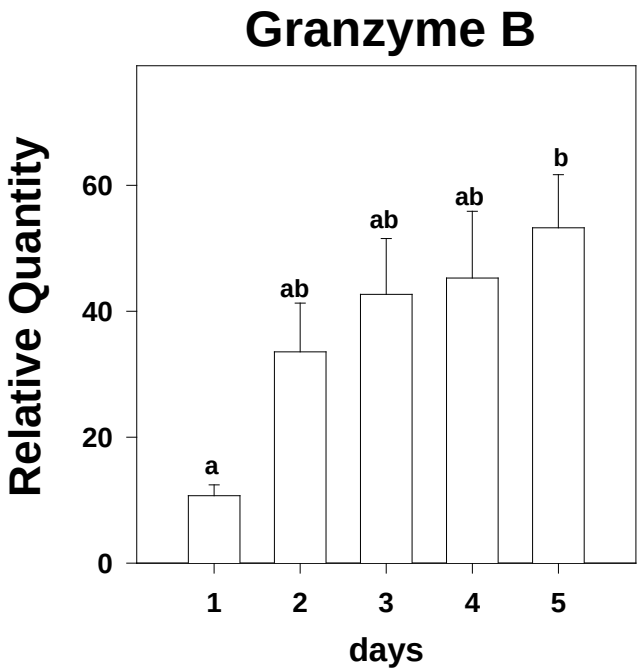
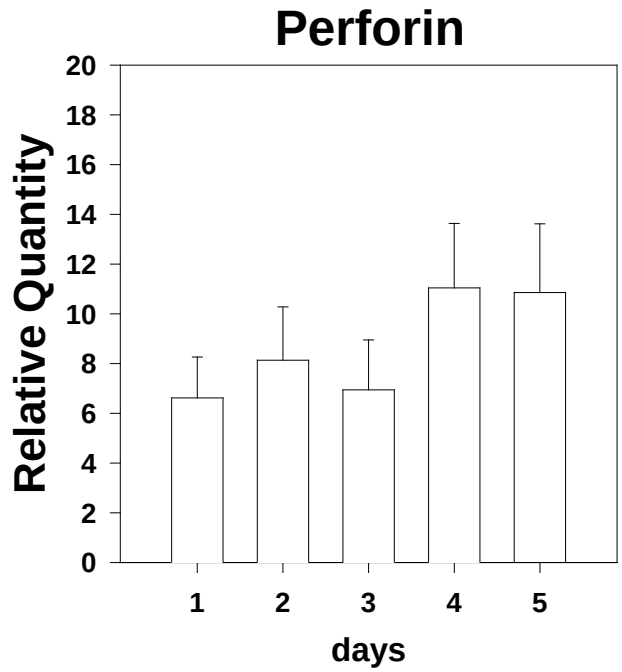


Figure 3. Dose-dependent induction of equine LAK activity by recombinant hIL-2. LAK cells were generated using 20, 200 and 2000 units/ml recombinant hIL-2. After 96h, LAK cells were co-cultured with EqT8888 cells at E: T ratio 10 for 3h. Flow cytometric assay was used to determine apoptosis. All determinations were performed in triplicate.

(a)


LAK activity
% cytotoxiciy
40
30
20
10
0
a
a
b
c
c
1
2
3
4
5
days


Granzyme B
Relative Quantity
60
40
20
0
a
ab
ab
ab
b
1
2
3
4
5
days


Perforin
Relative Quantity
20
18
16
14
12
10
8
6
4
2
0
1
2
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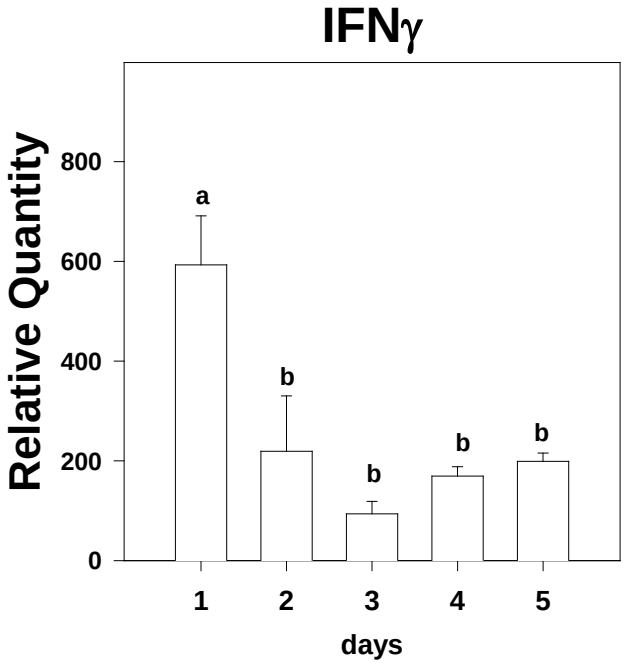
days


IFNγ
Relative Quantity
800
600
400
200
0
a
b
b
b
b
1
2
3
4
5
days

(b)

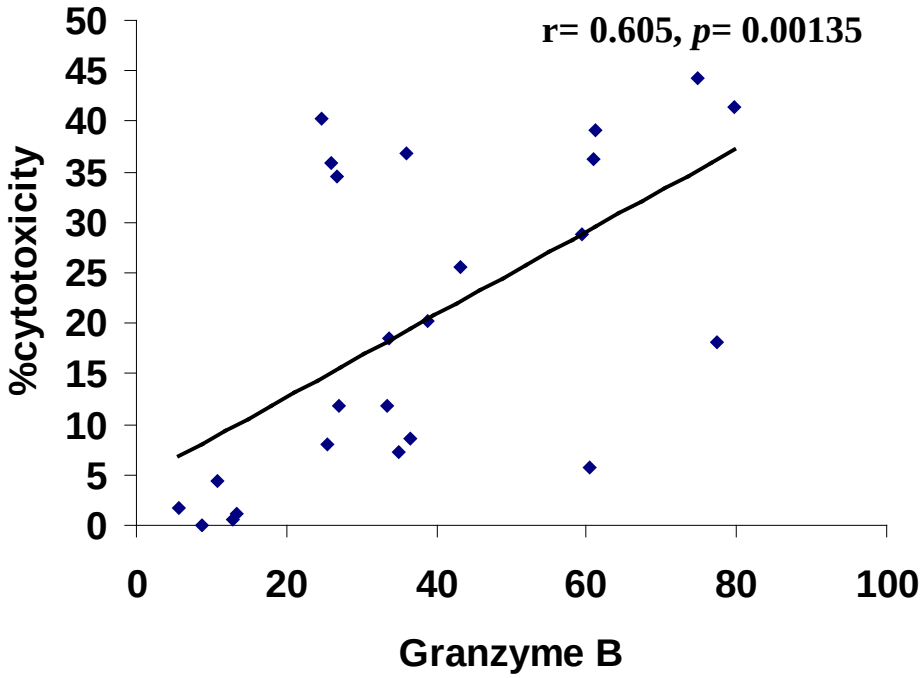
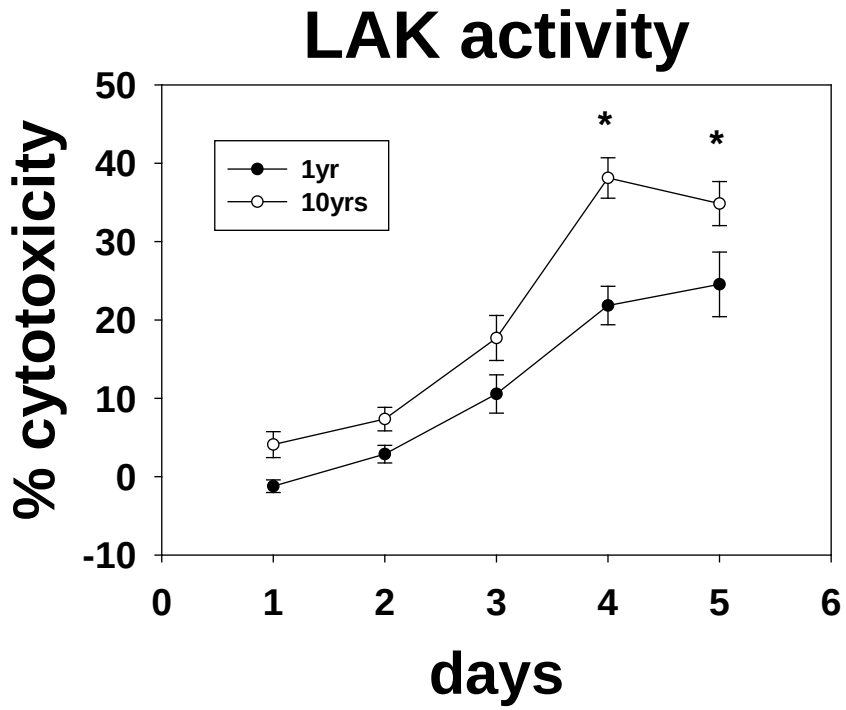


Figure 4. Comparison of LAK activity and gene expressions. (a) Temporal induction of LAK activity and mRNA expression in PBMC cultures. PBMC were incubated with 2000 U hIL-2 for various times for both flow cytometric assay of LAK activity and real-time PCR analysis of gene expression, as indicated. Flow cytometric determination of cytotoxicity was performed in triplicate. Means without a common letters (a,b,c) differ at $p<0.05$. (b) Granzyme B mRNA expression correlates with LAK activity. Relationships between LAK activity and granzyme B mRNA expression were assessed by Pearson Product Moment Correlation. Linear regression line, correlation coefficient (r), and *p* value is shown.



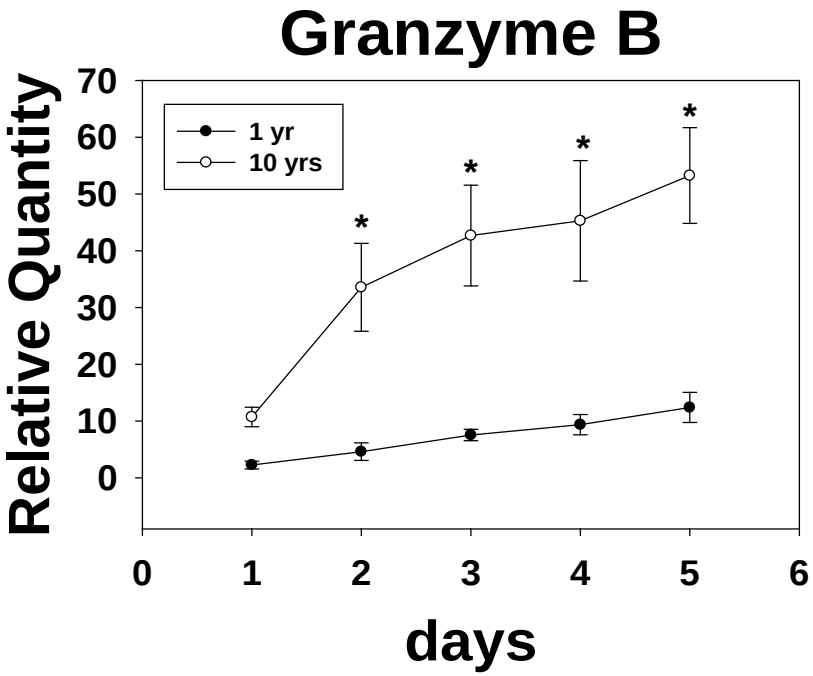


Figure 5. Younger horses exhibit reduced LAK activity and granzyme B mRNA expression. PBMC from 1 and 10 year old horses were cultured with 2000 units/ml human

recombinant IL-2 for 5 days. LAK activity, determined by flow cytometric assay on the days indicated. Granzyme B, perforin and IFNγ mRNA expression was determined by real-time PCR daily. Two-way RM-ANOVA was used to identify differences between age groups over time. The Holm-Sidak method was used to determine differences between age groups at each day. An * indicates significant difference at $p<0.05$.

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4. The 1st Class Scholarship, Northeast Normal University, China (2004,2005,2006)

5. The 1st Prize, National Biology League Matches at Province level, Hebei, China (2001)

PEER REVIEWED PUBLICATIONS

1. Quan-ping Su, De-zhong Wen, Qiong Yang, Yan-hui, Zhang, Chong Liu, Li Wang. Comparison of phage pV and KLH as vector in inducing the production of cytokines in C57BL/6J mice. Vaccine, 176, (2008), P 212-219

2. Liu, C., A. Betancourt, D. A. Cohen, A. A. Adams, L. Sun, and D. W. Horohov. Granzyme B- mRNA expression by equine lymphokine activated killer (LAK) cells is associated with the induction of apoptosis in target cells. Vet Immunol Immunopathol 143:108-15. (2011)

3. Sun, L., A. A. Adams, A. Betancourt, J. C. Stewart, C. Liu, and D. W. Horohov. The role of proliferation in the regulation of interferon gamma expression in foals. Developmental & Comparative Immunology. (2011)

4. Liu, C., F. R. Cook, et al. (2012). "The determination of in vivo envelope-specific cell-mediated immune responses in equine infectious anemia virus-infected ponies." Vet Immunol Immunopathol 148(3-4): 302-310.

5. Jodi K. Craigo, Corin Ezzelarab, Sheila J. Cook, Liu Chong, David Horohov, Charles J. Issel, and Ronald C. Montelaro " Mapping equine lentiviral envelope determinants of vaccine protection", PLOS One, Accepted (2013)

6. Liu, C., S. J. Cook, et al. " Epitope shifting of gp90-specific cellular immune responses in EIAV infected ponies" Journal of Virology, (in preparation)

7. Lingshuang Sun, Eric J. Oberst, Alejandra Betancourt, Don Cohen, Frank Cook, Tom Chambers, Chong Liu, David W. Horohov. The effect of environment on lung antigen presenting cells (APC) in foals. Vet Immunol Immunopathol ,(in preparation)

8. Jordan Dunham, Chong Liu and David W. Horohov. "Characterization of the immunological responses of horses to vaccine adjuvants." Vet Immunol Immunopathol, (in preparation)

ABSTRACTS/PRESENTATIONS:

1. 12/2009 Chong Liu, David W. Horohov. Establishment of a two-color flow cytometry assay for determination of lymphokine activated killer (LAK) activity in horses. Conference of research workers in animal diseases, Chicago, IL.

2. 5/2009 Chong Liu, Good markers for CTLs. Department of Veterinary Science. University of Kentucky.

3. 12/2010 Chong Liu, Sheila J. Cook, Amanda A. Adams. David W. Horohov. Development of a skin test to map equine infectious anemia virus (EIAV) envelope-

specific immune responses in vivo. Conference of research workers in animal diseases, Chicago, IL.

4. 10/2011 Chong Liu The maturation of equine infectious anemia virus (EIAV) envelope-specific immune responses in vivo. Department of Veterinary Science. University of Kentucky.

5. 12/2011 Jordon Dunham, Chong Liu, David. W. Horohov. Comparison of the ability of two different adjuvants to stimulate antigen presenting cells function in vivo. Conference of research workers in animal diseases, Chicago, IL.

6. 12/2011 Chong Liu, Sheila J. Cook, Jodi K. Craigo, Charles J. Issel, Ronald C. Montelaro, David W. Horohov. The maturation of equine infectious anemia virus (EIAV) envelope-specific immune responses in vivo after exposure to a live-attenuated vaccine. Conference of research workers in animal diseases, Chicago, IL.

7. 10/2012 C. Liu, S. J. Cook, J. K. Craigo, R. F. Cook, C. J. Issel, R.C. Montelaro, D.W. Horohov. Stability differences of envelope-specific T cells responses between newly EIAV infected and inaparent carrier horses. The Ninth International Equine Infectious Disease Conference, Lexington, KY.

8. 4/2013 Talia Henkle, Chong Liu, David W. Horohov. Comparison of immune gene expression between virulent EIAV and an attenuated vaccine strain. 27th NCUR, Wisconsin USA